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IN OCTOBER 1956 when I last spoke to Dr. Beaudette, he recalled that some years ago he and Dr. Hinshaw had seriously considered starting a journal of avian pathology. Then World War II intervened and the matter was dropped. Dr. Beaudette was enthusiastic about our plans to start Avian Diseases, agreed to serve on the editorial board and offered to assist in any way he could. His illness and untimely death came shortly afterward. It is fitting, then, that the first number of the new journal, Avian Diseases, be dedicated to his memory. It is also appropriate that Dr. Hinshaw, the partner in the projected journal years ago, should write the first and feature article on Dr. Beaudette's life and accomplishments.

The editors believe that a journal devoted to avian diseases is long overdue. A growing corps of poultry pathologists, striving to meet the needs of a rapidly expanding industry, should have a medium of communication dealing with its specialty. It is our intent that Avian Diseases will publish the findings of current research and help scientists assess the work of others without undue delay. The launching of a new, scientific journal is not undertaken lightly and it is with full realization of its obligation that the editorial board has embarked on this project. We hope to publish a journal of high quality with its usefulness and scope not limited by any geographic or political boundaries. With these aims the Board of Directors of the Cornell Veterinarian concurs and has agreed to act as publisher of Avian Diseases. The only requirement is that this journal be self-sufficient. We ask for the support of our colleagues everywhere.

P. P. LEVINE

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Any member of the Editorial Staff will be glad to consider manuscripts covering subject matter in the general field of avian diseases. Manuscripts should be typewritten and double-spaced. Authors are urged to consult recent copies of Avian Diseases or the Cornell Veterinarian for the style used in this journal.

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FRED ROBERT BEAUDETTE

FRED ROBERT BEAUDETTE
DISTINGUISHED AVIAN MICROBIOLOGIST AND PATHOLOGIST
1897 - 1957

THE untimely death of Fred R. Beaudette on January 17, 1957 at the still productive age of 59 has deprived the scientific world, and especially the veterinary profession and the poultry industry of an outstanding expert in the field of poultry disease research. He was born in Wichita, Kansas on April 15, 1897. In 1922 he married Velva Rader of Caney, Kansas. They raised three sons, Robert R., New York City, N. Y.; John H., Poughkeepsie, N. Y.; and Thomas R., Denver, Colorado. This tribute is dedicated to Fred by his many friends and colleagues in his chosen field of reseach—avian diseases.

Dr. Beaudette was one of the pioneers of a second generation of American avian veterinary microbiologists; the first generation included such men as V. A. Moore, Leo F. Rettger, L. D. Bushnell, Cooper Curtice, A. R. Ward, H. W. Graybill and E. E. Tyzzer. Although Fred, or "Red" as many of his colleagues knew him, died prematurely, his contributions to the poultry industry, veterinary medicine and biological science in general were those that would honor a much older man. He started his career in the field of avian veterinary science and teaching in 1919 after receiving his degree of Doctor of Veterinary Medicine from Kansas State College at the age of 22. He attributed his early interest, inspirations and training in the field of poultry diseases to Dr. L. D. Bushnell, who first

hired him as Instructor in Bacteriology in the Kansas State College Department of Bacteriology and as Poultry Bacteriologist in the Kansas Agricultural Experiment Station. In addition to aiding in the teaching of veterinary bacteriology, he initiated a course in poultry bacteriology for poultry majors, and had charge of the poultry disease diagnostic laboratory which furnished ample material for research problems. This latter is evidenced by the number of papers published by him or in cooperation with Dr. Bushnell in the four years (1919 to 1923) he was a member of the staff of the K. S. C.

In 1921, Beaudette was promoted to Assistant Professor, an assignment he held when he left Kansas in June 1923 to accept a position as Assistant Professor at Rutgers University. In 1925, Rutgers University promoted him to Associate Professor and in 1929 to Professor, the position he held until his death. From 1923 to his death, he was also Poultry Pathologist in the New Jersey Agricultural Experiment Station.

Dr. Beaudette was the first veterinarian to be employed by the New Jersey Agricultural Experiment Station to do research on avian diseases. It was through the efforts of Dr. A. D. Goldhaft, a New Jersey veterinarian who started specializing in poultry diseases in 1914, and a group of poultry industry leaders, that Beaudette was hired in 1923. According to Dr. Goldhaft, the initial interest in this field of research in New Jersey followed the loss of 50,000 ducks in 1917 by the Huey Duck Farm from an undiagnosed cause but it took six years to get funds and find a qualified man for the job. In 1925, through efforts of a similar group in Vineland, a branch of the state laboratory at New Brunswick was established. Dr. James Black, Kansas State Agricultural College Extension Veterinarian and a former student of Drs. Bushnell and Beaudette was hired to take charge of this laboratory, a position he held until his death in 1955. In 1927, further expansion of the research work at the New Brunswick laboratory was made possible by the employment of Mr. C. B. Hudson, a bacteriologist also trained by Dr. Bushnell of Kansas State College. Thus the team of Beaudette, Black and Hudson was established.

Until 1954, poultry disease research in New Jersey was a function of the Department of Poultry Husbandry. In 1954, Rutgers University established a Department of Animal Pathology and recognized Beaudette's years of service and professional ability by appointing him the first chairman of the new department. Poultry pathology was transferred to the new department and continued to be Beaudette's field of research and teaching in addition to administration of other activities in the field of veterinary science.

Fred Beaudette won many honors in recognition of his scientific accomplishments and for services rendered to the poultry industries of not only his adopted state of New Jersey but of the United States and the world at large. These honors included the Borden Award in Poultry Science in 1944; a citation in 1948 by the New Jersey State Grange on his 25th Anniversary at Rutgers University; the "Golden Egg" honor with a $1,500 check and a television set by the New Jersey Poultry Association in 1949; the Tom Newman Award of the Great Britain Poultry Association in 1950 for being the "most outstanding worker in poultry husbandry research"; and, in 1951 election as a Fellow of Poultry Science Association. He was a member of a number of scientific societies and served on the editorial staffs of at least two Poultry Science Associations (1946 to 1951) and American Veterinary Medical Association (1947 to 1949).

In 1951 he was granted an honorary degree of Sc.D. by Rutgers University. In 1955, he was again honored by Rutgers with a Distinguished Research Citation for his "long, industrious and fruitful labors to enlarge man's knowledge of avian diseases."

A final recognition for service was given him posthumously on January 22, 1957 by the New Jersey State Board of Agriculture. On that day Dr. Beaudette, along with three other New Jersey Agricultural leaders, Irving T. Gumb, Charles W. M. Hess and Grace M. Ziegler were given "Citations for Distinguished Service to New Jersey Agriculture." The citation of Fred R. Beaudette is quoted below:

"New Jersey is proud of you as an adopted son, and

grateful that you chose our State in which to pursue your career as a poultry pathologist for more than one-third of a century.

"Our older poultrymen well remember how you lifted a great burden from their shoulders in 1933 when you emerged from your laboratory with the first vaccine for the prevention of laryngotracheitis, a great poultry scourge of that day.

"Again, in 1944, when a new disease threatened our flocks and heavy economic losses were being sustained, you were the first to identify the causative virus, thereby opening the doors for research leading to vaccines and preventive measures for Newcastle disease.

"You have been an effective and sympathetic teacher not only at Rutgers University but as a lecturer throughout the nation and in foreign lands. You have given freely of your time and your great talents to instruct thousands of practical farmers in methods of combatting avian diseases, thus increasing the production efficiency of our flocks and making possible the expansion of the poultry industry. The consuming public as well as agriculture has profited immeasurably.

"Uncounted beneficiaries of your outstanding achievements and men of the world of science and education acclaim this award by the State Board of Agriculture of the CITATION FOR DISTINGUISHED SERVICE TO NEW JERSEY AGRICULTURE."

I feel that I am well acquainted with Fred's professional background because it was my good fortune to be employed as his successor when he left Kansas State College in 1923. It was my first job after college and a tough one to fill, because Fred had left a lasting impression on students and staff alike as a brilliant student himself, an excellent teacher and was well established as a poultry disease expert. I shared a room with Dr. Jim Black, one of his former students, in the home of a family where Fred himself had roomed for several years before he married in 1922. It was not long before I realized what big shoes I had to fill because wherever I turned, I found

people extolling the virtues of "Red" Beaudette. These included daily praises and reminiscences by both my landlady and my roommate.

Since Fred had left before I arrived to take over his former duties, I had no chance to see what he looked like or to obtain his advice regarding my duties. He had the reputation as a teacher and as a public speaker of never using a note of any kind and consequently left no lecture notes for me. Dr. L. D. Bushnell, my chief, was of little help because he was of the school who believed in letting his staff members "find" themselves. Luckily, I inherited with my job a student assistant of "Red's" a senior, Charles J. Coon now of Sioux Falls, S. D. Coon was as old or older than I was and immediately assumed a role of an advisor. Without his guidance I'm sure I would never have survived my first year as Beaudette's successor. I might add that Dr. Bushnell himself was an excellent boss and teacher who never really let a young staff member "sink," and probably guided me through Charlie Coon, more than I realized at the time. Therefore, I feel that I learned to know Fred R. Beaudette by the sweat of my brow, and came to have great respect for him long before I met him personally for the first time two years after I became his successor.

The Fred R. Beaudette bibliography of 146 titles published as part of this tribute to him has been compiled through the cooperation of his former staff members at Rutgers University, Dr. A. D. Goldhaft and Professor V. D. Foltz of the Department of Bacteriology, Kansas State College. No doubt many popular press references have been overlooked, but every effort was made to include a complete list of his scientific publications. Attention should be called to the fact that this bibliography records only those papers authored or coauthored by Beaudette. An almost equally impressive list could be compiled of publications coming from his Rutgers' laboratories but authored by his colleagues working under him. Fred would want to give due credit for research accomplished by his many students and staff members, including the late Jim Black, C. B. Hudson, J. A. Bivins, A. Komarov, Barbara R. Miller, Helen E. Burd, D. C. Tudor, O. Schwabe and V. R. Kaschula.

Beaudette excelled as a writer of review type papers and was especially noted for his complete serial reviews on equine encephalomyelitis and Newcastle disease. A paper "Twenty Years of Progress in Immunization Against Virus Diseases of Birds" published in 1949 is another example. The book "Psittacosis-Diagnosis, Epidemiology and Control" edited by him is a proceedings of a symposium sponsored by Rutgers University in 1953 under his guidance and is further evidence of his ability to compile literature in a single text for reference purposes. For a number of years he edited a yearly review on poultry disease compiled by the cooperative efforts of workers in the field and the Northeastern Conference of Laboratory Workers in Pullorum Disease Control. This review was widely circulated and became a standard source of poultry disease references. One could always be sure that even a case report of a single outbreak published by Beaudette would contain a complete background survey of both English and foreign literature. Dr. Beaudette's ability to read at least six foreign languages was utilized to good advantage in preparation of his reviews. His collection of reprints of papers dealing with poultry diseases is probably the most complete in the world.

He believed in fullest cooperation with scientists in allied fields of both institutional and commercial research. An outstanding example is the cooperative efforts of Doctors Webster, Hughes and Pritchard of Rockefeller Institute of Medical Research in a complete study of fowl cholera. His research with Dr. Waksman and his staff in an effort to find antiviral agents is another example. One of the two strains of *Streptomyces griseus* used for Waksman's early studies on streptomycin is reported to have been isolated by Doris Jones from the throat of a chicken in Beaudette's laboratory. The development of a chicken embryo propagated infectious laryngotracheitis vaccine and his close personal supervision and control of its manufacture in cooperation with one of the pioneer laboratories producing poultry biologics until it was thoroughly field tested was an unusual but apparently successful university research-commercial production arrangement.

Beaudette was such a popular speaker on poultry disease subjects, that he had the reputation at one time in his career of spending as much time traveling in other states as at his own headquarters. His talents along these lines, however, gave his department at Rutgers University a national reputation for its poultry disease research. His fame as a speaker was not confined to the United States since his name has appeared frequently on programs in Europe. In 1954 he answered an urgent appeal from the Brazilian Government to come to that country to help its veterinarians and farmers develop a program to control the first epizootic of Newcastle disease to hit that country. In 1955, accompanied by his wife, he made a month-long flying tour of the African continent observing poultry pathology work in various countries, including Morocco, French West Africa, Belgian Congo, Union of South Africa, Portugese East Africa, Northern Rhodesia, Kenya and Israel.

One of the greatest tributes that can be paid to Beaudette was the respect the "dirt poultry farmer" had for him. The announcement of his name on any poultry program was a guarantee of a good attendance. This was not limited to farmer audiences. His schools for instructing veterinarians on poultry diseases were very popular in the early 1930's. His encyclopedic knowledge of his subject and his frankness in discussing poultry disease problems won him an enviable reputation. Many times his frankness irritated his audiences but they went away knowing exactly where he stood on any question.

He prided himself as a person who understood the applied as well as the fundamental phases of avian disease problems and could discuss them equally well with the veterinary practitioner, scientist, poultry farmer, farm advisor, hatcheryman, feed dealer and remedy salesman. His extensive bibliography illustrates this point. It will be noted that a great many of his writings were of a popular type. The wide variety of subjects and the thoroughness of their coverage demonstrates his wide interest in the field of his choice.

He was a virologist at heart and probably his second

interest was parasitology rather than bacteriology. If his research teams lacked support in any one area, it was in the field of histopathology. This, however, was until the past few years, a characteristic of many United States poultry disease research laboratories. In his earlier days, Beaudette believed that histopathological support was unessential to the solution of a major poultry disease problem. In recent years, he often admitted however that work in histopathology might have added even more to accomplishments from his laboratories.

The following excerpts from a commemorative statement made by Dr. William H. Martin, Dean of the New Jersey Agricultural Experiment Station, following his untimely passing make an especially apropriate closing paragraph to this tribute:

> "Dr. Beaudette has long ranked with the greatest of the distinguished scientists who have brought glory to the New Jersey Agricultural Experiment Station down through the years. A brilliant investigator, his discoveries in the field of poultry pathology have meant savings of many millions to the poultry industry of the nation, and indeed of the entire world.
>
> "To sum up, Dr. Beaudette's untimely death has taken from us a truly great man."

WILLIAM R. HINSHAW
Fort Detrick, Maryland

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THE BEAUDETTE VETERINARY HISTORY COLLECTION

AMONG the many avocational interests of Doctor Beaudette, one is deserving of particular notice because of the continuing influence it may be expected to have in the veterinary profession. This is the fabulous collection of old and rare books on veterinary medicine assembled by him and which has been acquired for the Historical Library of the College of Veterinary Medicine at Michigan State University. As a fitting monument to the man and his unique distinction as a veterinary bibliophile, these works will retain their identity as the Fred R. Beaudette Collection.

This collection, consisting of about 250 distinct works (some more than one volume), contains six items from before 1500, another 19 before 1600, and a total of 60 items published before 1700. Few such collections in existence contain such a wealth of early material. As might be expected, there are a number of items relating to the diseases of birds, including several extremely rare 16th and 17th century works on the diseases of falcons, and one on canaries. Together with books already held, the MSU Historical Library, including the Beaudette Collection, presently consists of more than 400 distinct works of which 80 are dated prior to 1700.

Such a collection, of course, serves an important function as a repository for the significant works which form the background of the veterinary profession. But more than this, it will be used to implement an active program of teaching, research and writing in the area of the history of veterinary medicine. In addition, as an outstanding collection on this aspect of the larger area of the history of civilization, it is anticipated that scholars from other areas may find source material here that is not readily available elsewhere. Rather than being buried in a vault, both the Beaudette Collection and the Historical Library as a whole will be on display—with

proper safeguards—in the Veterinary Medicine Reading Room at MSU, and both will be open to qualified scholars. The many friends of Doctor Beaudette are always welcome to drop in to see the collection whenever they may be in the vicinity.

J. F. Smithcors
Michigan State University

STRAIN DIFFERENCES OF PLEUROPNEUMONIALIKE ORGANISMS OF AVIAN ORIGIN

By H. E. ADLER, R. YAMAMOTO, and J. BERG
School of Veterinary Medicine
University of California, Davis, California

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PLEUROPNEUMONIALIKE organisms (PPLO) have been routinely isolated from the respiratory tract of chickens and turkeys. Since the studies of Nelson[11] and more recently Markham and Wong,[10] there has been some question concerning the role of this group of organisms as the cause of chronic respiratory disease (CRD). Reports from the various laboratories suggest that PPLO can cause either a mild disease or that the organisms are nonpathogens.[8,9] Serological studies[4,11] on a number of different strains isolated from so-called air sac infection showed that the organisms were antigenically similar. The observations to be presented

in this paper indicated that at least two different types of PPLO were present in chicken and turkey tissues. These types were different in serology and in pathogenicity for turkeys.

MATERIALS AND METHODS

Sterile cotton swabs were used to obtain specimens from the various sites in chickens and turkeys. The swabs were immediately placed in the selective enrichment broths for PPLO isolation, as previously described.[1] To avoid the possibility that the original four strains isolated were L-phase bacterial variants resulting from the use of penicillin in the original selective medium, the organisms were blind-passed in noninhibitory serum broth for 10 generations.[3]

In all of the chicken embryo studies the inoculations were made according to the procedures described by Cunningham.[2] The seventh culture passage was used for the initial inoculum for the egg studies with the strains isolated from the turbinate, cloaca, and mouth of chickens. A culture from the brain strain that had been subcultured through one broth passage was used in the chicken embryo pathogenicity studies.

Antigens were prepared from PPLO cultures that had been propagated in Difco PPLO broth enriched with 1 percent yeast hydrolysate* and adjusted to pH 7.8 before autoclaving. Ten percent horse serum was added aseptically to the cooled autoclaved broth. The strain designated "brain" was allowed to incubate for 6 days at 37 C. All other strains, as designated by site of location, were harvested at 2 days. After incubation the organisms were concentrated in an angle centrifuge at 10,200 rpm for 30 minutes. These organisms were standarized to 50× the opacity of the no. 1 McFarland density standard.

The same antigens were used for the ammonium sulfate sedimentation studies. The method of testing the various antigens by ammonium sulfate flocculation was essentially as described by Williams.[15] In addition, these antigens were used the day of harvest for the hemagglutination tests.

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* A product of Nutritional Biochemical Corporation, Cleveland, Ohio.

The antisera for the four strains of PPLO tested were prepared by inoculating rabbits six times intraperitoneally at 3-day intervals. Rabbits were bled 8 days after the last inoculation. The inocula were 1-ml portions of live culture suspended in buffered saline at a concentration of 50×. All antigens were washed three times by suspension in buffered saline and centrifugation at 10,200 rpm for 30 minutes. Previous experience had shown that this treatment would prevent the production of rabbit antibodies for foreign proteins.

Agglutination tests were conducted by a slide agglutination procedure using twofold dilutions of sera in buffered saline. The end point of the titrations was determined grossly by obvious agglutination. Antigen and serum controls were included at the start of each series of tests.

The methods of Gianforte *et. al.*[4] and Van Herick and Eaton[13] were used to determine the ability of the various strains to agglutinate chicken red blood cells.

For evaluation of the ability to utilize various carbohydrates, cultures were seeded into Difco phenol red broth enriched with sterile, Seitz-filtered 10 percent horse serum. These cultures were incubated at 38 C and examined for acid production at 8-hour intervals for 6 days. The substrates used were levulose, maltose, starch, dextrin, glucose, galactose, mannose, sucrose, lactose, dulcitol, and mannitol. These substrates were added at the 1.0 percent level before autoclaving for 10 minutes at 110 C.

For morphological studies of the PPLO, sedimented cultures or intact colonies were fixed and stained by the method of Klieneberger.[7]

To determine growth of the PPLO at 22 C, the organisms were incubated in a Cenco refrigerating incubator for 6 days. The plates were examined with a dissecting microscope at 100-diameter magnification.

RESULTS

A large number of PPLO strains were isolated from the various sites in and on the bird's body. Because of technical difficulties only a few typical strains were selected for the

intensive studies. One strain, designated "brain," was antigenically related to the known pathogenic strains. This strain was isolated from the brain of a turkey showing encephalitic symptoms. The mouth, cloaca, and turbinate strains were recovered from these sites in asymptomatic birds.

Examination of the stained preparations from each strain revealed that the brain strain consisted of well-defined coccoid forms that were maintained unchanged for 6 days of incubation. The other three strains showed coccoid forms for the first 10 hours of incubation. At 24 hours of incubation these cultures were composed of predominantly ring forms.

The results of cross-agglutination tests with these strains of PPLO may be seen in table I. It is apparent from these data that two serological types were present.

TABLE I

Results of cross-agglutination tests with four strains of PPLO of chicken and turkey origin

Antisera	Antigens			
Strain	Cloaca	Turbinate	Mouth	Brain
Cloaca	*320*	40	80	—
Turbinate	80	*160*	160	—
Mouth	20	40	*80*	—
Brain	—	—	—	*640*

— = no agglutination at 1:10.

Figures indicate the highest dilution at which agglutination occurred.

Table II shows the ammonium sulfate flocculation differences between the mouth, cloaca, and turbinate strains as compared to the strain recovered from the brain. This procedure was repeated three times with essentially the same results. The age of the antigen or stage of development of the PPLO did not seem to alter the ammonium sulfate

precipitation results. A few aged antigens that failed to give satisfactory agglutination tests were consistently flocculated at the same ammonium sulfate concentration regardless of the aging effect on agglutination.

The brain strain agglutinated washed chicken blood cells. Attempts to prepare a hemagglutinating antigen with the other three strains failed, even though the two different procedures[4,13] of preparing antigen were followed.

The biochemical tests showed that all strains produced acid and no gas from levulose, maltose, starch, dextrin,

TABLE II

Effect of various concentrations of ammonium sulfate on the flocculation of four strains of avian pleuropneumonialike organisms

$(NH_4)_2SO_4$ %	Strain and source of PPLO antigen with degree of turbidity*			
	Turkey brain	Chicken cloaca	Chicken mouth	Chicken turbinate
15	+	++	++	++
16	+	++	++	++
17	+	++	++	++
18	±	++	++	++
19	—	++	++	++
20	—	++	++	++
21	—	++	++	++
22	—	++	++	+
23	—	+	+	±
24	—	—	+	—
25	—	—	—	—
26	—	—	—	—
27	—	—	—	—
28	—	—	—	—

* — = clear supernate. ± = trace of turbidity. + = moderate turbidity. ++ = marked turbidity.

glucose, galactose, mannose, and sucrose. The three other substrates, lactose, dulcitol, and mannitol, were not utilized by any of the strains. There was one difference in fermentation reaction between the brain strain and the other three strains studied. The brain strain failed to attack sucrose in less than 80 hours. Sucrose was fermented within 24 hours by the cloaca, mouth, and turbinate strains. All strains failed to grow either at 22 C or in the absence of serum.

The cloaca, mouth, and turbinate strains were adapted to produce uniform embryo mortality after the fifth generation in chicken embryos. The turbinate strain killed all 10 embryos within 6 days postinoculation. With the mouth and cloacal strains, all 10 embryos succumbed to infection by the 8th and 10th day following inoculation. The brain strain produced typical embryo mortality following eight blind passages in chicken embryos. The pattern of embryo mortality with these strains was similar to one reported by Hoyt.[6]

Three 4-week-old broad-breasted bronze turkeys that had been reared in strict isolation were used for each strain to test the ability to produce sinusitis. The source of PPLO inocula were the egg-adapted PPLO of the previous study. Each poult was inoculated in the right infraorbital sinus with 0.5 ml of undiluted PPLO yolk. The birds were observed daily for 3 weeks. The brain strain produced visible swelling of the sinuses in 8 days, and the other strains failed to infect the birds. Agglutination tests with the sera from the poults infected with the brain strain agglutinated only the homologous antigen. The sera from the other poults did not agglutinate their homologous antigen.

Eleven other PPLO strains isolated during the course of this study were evaluated by the agglutination test using the four antisera and the ammonium sulfate flocculation procedure. For convenience of identification, all antigen from strains that agglutinated with the brain strain immune serum were placed in group I. Ammonium sulfate flocculation levels similar to those of the brain strain also placed a strain in group I. The strains that had the characteristics of the other PPLO were placed in group II.

A majority of the strains seemed to fall into group II on the basis of the ammonium sulfate flocculation and agglutination procedures (table III). Further studies with the PPLO isolated from the turkey air sacs and the chicken pericardial fluid showed that these two strains were not serologically related to either group. Both of these strains failed to infect turkeys by intrasinusoidal inoculation.

Morphologically all cultures of strains classed in group II were composed of a large number of ring forms at the 2nd day of incubation.

TABLE III

Serological and ammonium sulfate identification of pleuropneumonialike organism strains isolated from chickens and turkeys

Source of strain	Identification procedure	
	Agglutination	Flocculation
Turkey air sac	—*	I*
Turkey mouth	II*	II
Turkey oviduct	II	II
Turkey ovary	I	I
Turkey turbinate	—	II
Chicken trachea	—	II
Chicken trachea	II	II
Rooster semen† (cloaca)	II	II
Rooster semen (cloaca)	II	II
Rooster semen (cloaca)	II	—
Chicken pericardialsac	—	—

* — = unclassified by procedure used. I = Group I serologically or by flocculation. II = Group II serologically or by flocculation. † = semen obtained by milking procedure.

DISCUSSION

The evidence of the presence of commensal or saprophytic PPLO in poultry tissues poses a difficult problem for the poultry pathologist. Isolation of PPLO as a routine practice

has not been readily accepted in poultry pathology laboratories because of the fastidious growth requirements of this group of organisms. In the future, isolations of PPLO will be further complicated by the necessity of typing these organisms by specific means into either pathogenic or other strains. Apparently the pathogenic strains are serologically related and can thus be distinguished from the other organisms.

Some strains of PPLO are not satisfactory for antigen production. In these instances the ammonium sulfate flocculation procedure would have value as an aid for the differentiation of strains. The fermentation of sucrose with the production of acid within 48 hours by the nonpathogenic strains would provide contributory information for the classification of strains on the basis of this report and that of Grumbles *et al.*[5] The tendency of the nonpathogens to develop ring forms of the organism within 24 hours of incubation in an enriched horse serum medium also provides useful presumptive evidence for classification in that group.

The commensal or saprophytic strains did not grow in the absence of serum or at 22 C. They, therefore, did not fit the criteria for saprophytic strains of PPLO suggested by Sabin.[12]

In view of the presence of the apparently nonpathogenic PPLO in the respiratory tract and genital tract, further work is required to determine the role of these organisms in respiratory disease and egg transmission.

SUMMARY

At least two different serological and pathological types of pleuro-pneumonialike organisms were present in chicken and turkey tissues. These two groups were primarily differentiated by agglutination and ammonium sulfate flocculation procedures.

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BLINDNESS IN TURKEYS DUE TO GRANULOMATOUS CHORIORETINITIS*

By L. Z. SAUNDERS and E. N. MOORE

Brookhaven National Laboratory,† Upton, New York, and Ohio Agricultural Experiment Station, Wooster, Ohio

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IN SEPTEMBER 1950 a 10-week-old White Holland male turkey was examined because of blindness which the owner had noted when it was 3 weeks old. The most obvious finding was that the left eye was smaller than the right one. During the subsequent 4 months several additional birds (including two bronze turkeys) in the flock became blind, and by January 1951 there were 15 affected birds, 8 males and 7 females out of a total of 300. The condition to be described was bilateral in three of the birds and unilateral in the others.

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* This study was commenced while the authors were in the Department of Pathology and Bacteriology of the New York State Veterinary College.

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In January 1951, when ophathalmoscopic examination was carried out, the 15 affected turkeys varied in age from 6 to 7 months. All had become affected by the time they were 5 weeks old. They were raised to maturity by one of us (E. N. M.); none recovered spontaneously. The gross and microscopic findings were similar in all of the affected birds and will be described in summary form. The affected eyes were smaller than normal and the corneas were flattened or concave. In most cases the cornea was opaque or else the lens was cataractous, preventing an ophthalmoscopic view of the fundus. The pupil of affected eyes did not repsond to light, but in unilateral cases the crossed pupillary reflex was sometimes present in the normal eye. The turkeys would follow the movements of the examiner's finger with the normal eye but not with the affected eye in unilateral cases. In some birds shreds of exudate could be seen in the anterior chamber, but there was no exudate on the conjunctivae.

PATHOLOGY

Although the condition superficially resembled microphthalmia, postmortem examination of four birds revealed that the eyelids, lacrimal glands, and bony orbits of the abnormally small eyes were the same size as those of normal eyes. The extraocular muscles dissected out from four affected eyes appeared normal in number and size. No lesions were found in the viscera. The abnormal eyes when dissected free of their muscles and orbital fat appeared reduced in diameter (figure 1) and in the anteroposterior dimension (figure 2). They were wrinkled around the circumference of the globe, and the corneas were flattened or concave (figure 2). The eyes were soft in consistency, and on palpation one gained the impression that the fluid had been withdrawn from the globe until it had collapsed. The eyes were fixed in aqueous formalin and embedded in celloidin. Paraffin sections of four turkey eyes affected with ocular aspergillosis and celloidin sections of four normal turkey eyes were available for comparison.

Histologically the eyes appeared collapsed, and in advanced cases the lens, vitreous, and retina had been destroyed.

Because of the collapse the region of the optic disc was almost in contact with the cornea (figure 5). It was necessary to study earlier stages (figure 4) to determine the pathogenesis of the condition. In these the choroid, retina, pecten, and lens were found to be primarily or secondarily involved in a

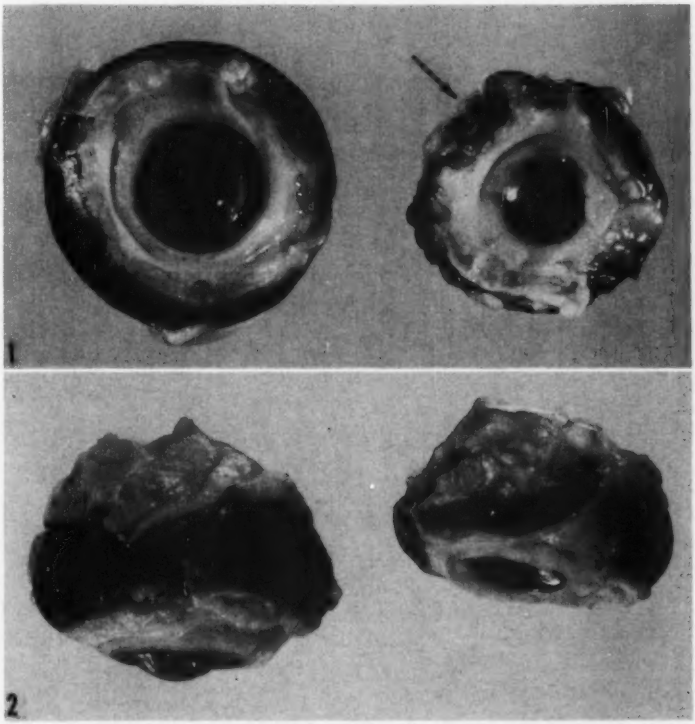

Fig. 1. Formalin-fixed eyes from a case of unilateral blindness. The arrow points to the wrinkling that was present in affected eyes.

Fig. 2. A dorsal view of the same eyes showing collapse of the cornea and decrease in the anteroposterior dimension.

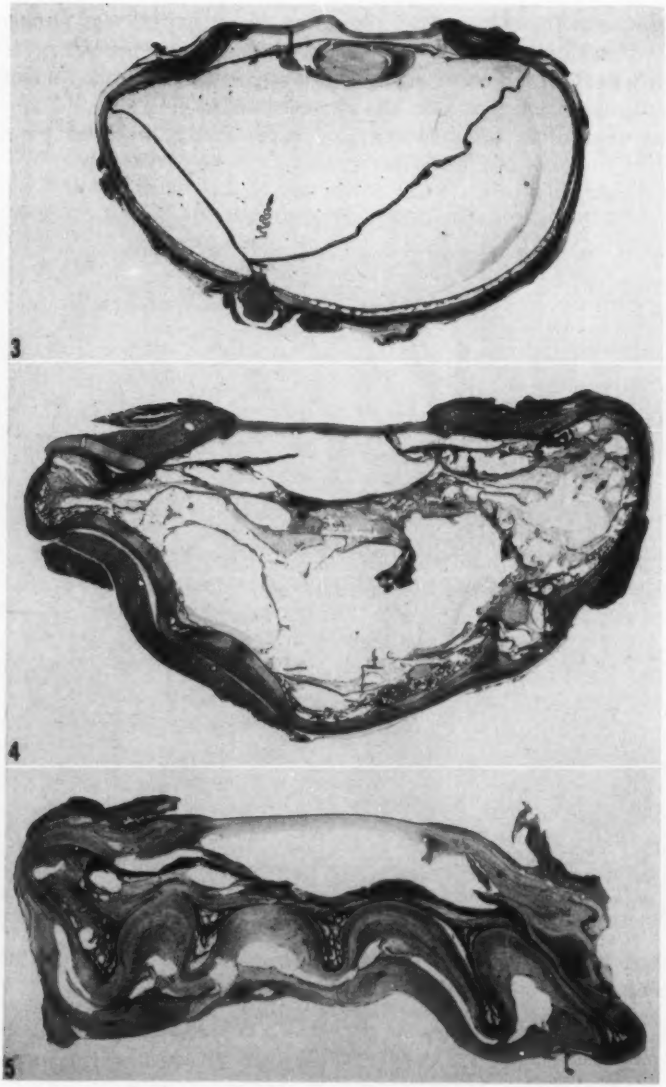

Fig. 3. Celloidin section of a normal turkey eye. (The wrinkling of the cornea and detachment of the retina are processing artifacts.)

Fig. 4. Chorioretinitis with destruction of vitreous and lens and beginning collapse of the globe.

Fig. 5. Chronic chorioretinitis in which the sclera is thrown into folds and the globe is completely collapsed.

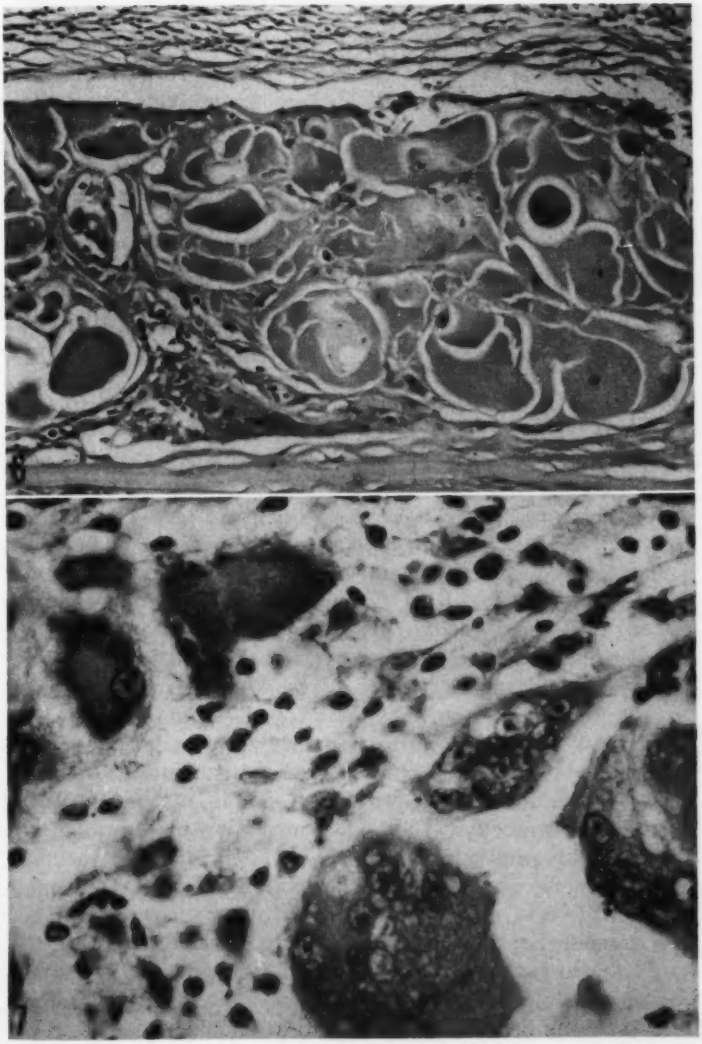

Fig. 6. Degeneration and fragmentation of the lens, with loss of its capsule, and invasion of the lens protein by inflammatory cells.

Fig. 7. Exudate in the posterior chamber containing giant cells which have phagocytosed melanin particles.

granulomatous inflammation. The posterior chamber was filled with exudate, which consisted of monoculear phagocytes, heterophils, lens protein (figure 6), necrotic retinal cells, and rod-like particles of melanin. Numerous giant cells were present (figure 7), and they contained phagocytosed particles of bacillary-shaped melanin released from the pigment epithelial layer. No bacteria or protozoa were seen in sections stained by the hematoxylin-eosin, Brown and Brenn, or Gram methods. In eyes in which the condition had been present longer there were more and larger giant cells, small clumps of mononuclear phagocytes, and disappearance of the heterophils seen in the earlier stages. Synechiae were present between the posterior surface of the iris and the exudate in the posterior chamber. The corneas showed beginning capillary proliferation into the tunica propria and adhesion of exudate to Descemet's membrane.

DISCUSSION

The impression gained from the histologic examination was that the lesions were chiefly a chorioretinitis. There did not appear to be any primary inflammation in the cornea, iris, or ciliary body, although the latter two organs were congested and surrounded by exudate. This created adhesions between many of the ciliary processes, but no inflammatory cells were seen within the processes. Thus the most striking gross lesion, that of shrinkage, can probably be explained on the basis of reduced intraocular pressure. Although the source of the exudate was the choroid and retina, once the ciliary body became involved it would be unable to continue production of intraocular fluid at a normal rate. As the fluid drained away and was not replaced, a drop in intraocular fluid pressure would occur. The eye depends on the maintenance of this pressure within rather narrow limits for both normal shape and normal function. If the drainage of the fluid is retarded, glaucoma results. Conversely, if the formation of the fluid is retarded, ophthalmomalacia, i.e., softening and shrinking, is the result. A similar shrinking of the globe, which does not, however, reach the extent seen here, occurs

in the chronic stages of periodic ophthalmia of horses.

In the absence of *Toxoplasma*, the granulomatous nature of the inflammation was suggestive of tuberculosis or of a fungus infection. No fungi were seen in the sections, and the lession did not resemble the ones in our cases of intraocular aspergillosis. Intraocular tuberculosis in animals is almost invariably secondary to pulmonary or gastrointestinal infection, neither of which were present in our birds. The flock as a whole was also free of tuberculosis, as determined at the time of slaughter. Histologic studies were not made until 1953, and since we did not suspect the possibility of bacterial infection while fresh tissue was available, no cultures were made on blood agar. In 1953 the same condition was found by one of us (E. N. M.) in 12 adult birds in a large bronze-turkey flock in Ohio. The lesions were identical to those in the New York outbreak, but this time, although cultures were made from the eyes, no growth was obtained on blood agar. (In the original outbreak cultures had been made only on Sabouraud's agar, and they were incubated at room temperature since aspergillosis had been suspected.) Eyes from the Ohio flock were embedded in paraffin, which provided an opportunity to make acid-fast stains of the sections; however, no acid-fast organisms were seen.

In 1950, with the possibility in mind of a hereditary predisposition to the disease, we had held some affected birds over until they reached breeding age and hatched some eggs from them. No blindness appeared in the resulting poults. The condition is apparently not contagious either, since no blindness appeared in the other turkeys which were in intimate pen contact with the affected breeding stock.

Apart from tuberculosis, another infectious disease of birds characterized by granuloma formation is the coli-granuloma first described in chickens by Hjärre and Wramby.[4] The causative organism was a mucoid strain of *Escherichia coli*, and the lesions were chiefly in the gastrointestinal tract, liver, and spleen. Köhler, who also studied the disease in chickens, found cases with lesions in the kidneys, muscles, pancreas, skin, oviducts, lungs, and air sacs.[5] Schofield subsequently

found the same condition in turkeys,[7] and it was also seen in this species by Savage and Isa.[6] None of these authors reported eye lesions, but the possibility that we are dealing with an ocular form of Hjärre and Wramby's disease cannot be ruled out until there is an opportunity to do more extensive bacteriologic study on fresh cases.

The lesions were somewhat similar to those of sympathetic ophthalmia of man, where phagocytosis of melanin by epithelioid cells is a characteristic feature.[9] According to Woods,[9] the disease is of unknown etiology, but since one of the workers he quotes postulated a rickettsial etiology, we stained paraffin sections from the Ohio cases by the Giemsa method. No rickettsia or any other organisms were seen in these sections. The severity of the inflammation was no doubt augmented by rupture of the lens capsule and release of its protein into the posterior chamber, since lens protein is known to elicit this reaction. However, we were unable to determine what had initiated the inflammation that led to the lens damage. In spite of the phagocytosis of melanin, we do not believe that the giant cells were elicited by the melanin particles, since this substance is not known to cause a foreign body inflammation. It seems more likely that the phagocytes were stimulated by some unidentified infectious agent, and once having arrived on the scene of the tissue destruction, they continued to ingest any available detritus. Wilder[8] has reported such phagocytosis of disrupted melanin particles in human chorioretinitis due to *Toxoplasma*.

Evans, Bruner, and Peckham[2] described an infectious blindness in chicks in New York which was associated with the presence of *Salmonella typhimurium* in affected eyes. The condition resembled the one in our turkeys in that it appeared in young birds, was characterized by inflammation of the intraocular structures, and resulted in eventual shrinking of the eyeball. It differed from the one in our turkeys in that it was characterized by increased mortality in the chicks and failure of the survivors to make normal gains; the *Salmonella* organism was isolated by Evans *et al.* from visceral organs as well as from affected eyes. They cited a

personal communication from Angstrom, who had observed blindness in turkey poults affected with paracolon infection. The lesion in our cases could perhaps be due to *Salmonella* or paracolon infection; however, both of these organisms are easily grown aerobes, and our failure to culture them from the Ohio birds would suggest that neither of them was present. It is possible, of course, that an organism could have been present in the early, acute stage of the inflammation and could have died out by the time we obtained the eyes. The *Salmonella* isolated by Evans *et al.* did not produce the eye lesion under experimental conditions. This suggests the possibility that its presence in the eye was due to its proved concurrent presence in the blood stream, and that the eye lesion itself was due to some other, unknown cause. That this possibility occurred to the authors may be inferred from the cautious wording of the title of their paper.[2]

Hinshaw and McNeil[3] also mentioned that eye lesions occurred in some types of paracolon infection in turkeys but gave no further details, so that we have nothing with which to compare the present condition. The keratoconjunctivitis described by Bierer[1] was characterized by considerable exudate under the lids. Its cause is not known, but it is anatomically quite different from the present condition in which there was no conjunctivitis and in which the cornea was only involved secondarily and from within.

SUMMARY

An outbreak of blindness affecting 15 birds occurred in a flock of 300 turkeys in New York State; 12 other cases were seen 4 years later in a large Ohio flock of 1,000 birds. The eyes were grossly collapsed and shrunken, and, histologically, lesions of granulomatous chorioretinitis were present. No organisms were cultured from the affected eyes nor were any seen in tissue sections. The lesions did not appear in the offspring of affected birds nor in turkeys penned with the affected birds. The etiology of the disease was not determined. On the basis of the lesions it appears to be a

hitherto-undescribed condition in this species. A somewhat similar inflammation, the cause of which is equally obscure, has been described in sympathetic ophthalmia of man.

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ESCHERICHIA COLI INFECTION OF THE CHICKEN EYE

By W. B. GROSS

Virginia Agricultural Experiment Station, Blacksburg

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IN THE COURSE of experimental work concerning a type of *Escherichia coli* infection characterized by pericarditis and perihepatitis,[3] an eye infection was sometimes noted. This eye infection was characterized by cloudiness in the anterior chamber without damage to the outer layers of the cornea. Bacteriological examination of the anterior chamber revealed pure cultures of *E. coli*. Unsuccessful attempts

were made to demonstrate PPLO in the chambers of the infected eyes by inoculating contents into turkey infraorbital sinuses and PPLO broth media. Several birds from flocks having a disease characterized by pericarditis, periphepatitis, and aerosacculitis were observed with this eye condition. Similar eye infections have been reported caused by Arizona paracolon organisms[2] and *Salmonella typhimurium*.[1]

EXPERIMENTAL PROCEDURE

The experimental eye infection was always associated with a severe *E. coli* bacteremia. The bacteremia resulted from the inoculation of certain types of *E. coli* alone or with yolk-propagated PPLO into the lesser abdominal air sacs of 3-to-9-week-old chickens. The birds were sacrificed and their eyes were fixed in Bouin's fluid, dehydrated with ethyl alcohol, and embedded in paraffin. Sections were stained with hematoxylin-eosin. Microscopic examination was made of stained sections from many of the infected eyes at various times after inoculation. Because the progress of infection varied greatly from bird to bird, time intervals will not be used in the description of these lesions, and because most birds with eye lesions died within 96 hours after inoculation, many more early than late lesions were available for examination.

RESULTS

The eye infection occurred in about 2 percent of the inoculated birds and was almost always unilateral. Between 20 and 90 percent of the birds with *E. coli* bacteremia died between the 1st and 4th day after inoculation, with only a few deaths occurring after the 6th day. Most of the birds which developed the eye infection died within 4 days after inoculation, but some lived for over a week and a few recovered.

The first symptoms occurred 24 to 48 hours after inoculation and consisted of a blindness associated with photophobia and a hypopyon. At first the gray deposit gravitated to the lower portion of the anterior chamber but soon became fixed in position. After 48 hours the greater portion of the anterior chamber often became filled with a cloudy fibrinous material. After several days the deposit usually obscured the iris and pupil. (See figure 1 showing an infected eye.)

Fig. 1. Left eye of an experimentally infected chicken 6 days after inoculation into the air sac. × 6.

The earliest microscopic lesions observed consisted of fibrin and a cellular infiltration in the anterior chamber. At first the heterophils were more common in the exudate, but later the mononuclear phagocytes became more prominent. Many heterophils and mononuclear cells infiltrated the ciliary body. The iris was somewhat edematous and hyperemic and contained a few mononuclear phagocytes. Figure 2 shows microscopic lesions of the anterior chamber and ciliary body. The outer surface of the cornea was never found to be involved. In later stages there was sometimes slight vascularization and mononuclear infiltration of the inner margin of the cornea.

The earliest lesions of the choroid consisted of an infiltra-

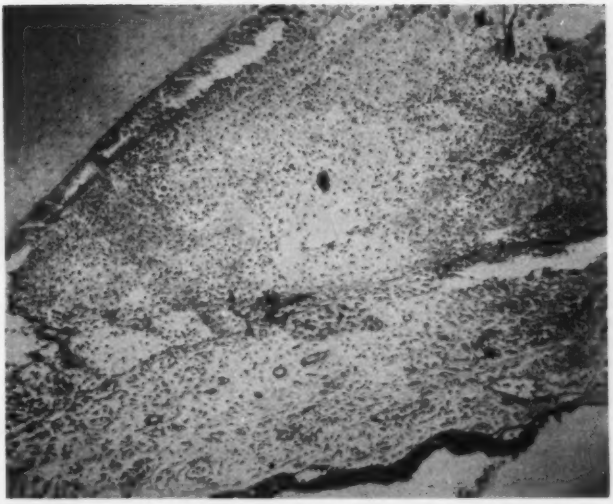

Fig. 2. Heterophil and mononuclear exudate in anterior chamber. Edema, hyperemia, and mononuclear infiltration of iris. × 100.

tion by heterophils and a few mononuclear phagocytes. The choroid thickness increased from a normal of about 18 microns to about 90 microns. As the inflammation progressed, there was edema and hyperemia and the mononuclear phagocytes became much more prominent. The retina soon became degenerate and later disappeared. When the lesions had completely developed, the choroid was sometimes over 900 microns thick. Figures 3 and 4 show the choroid lesions.

Within the eye granulomas developed in the ciliary body choroid, anterior chamber, and vitreous. At first, the lining of the granulomas consisted of heterophils and mononuclears, while later the lining was composed of giant cells and mononuclear phagocytes. In many eyes the entire vitreous became necrotic with an inflammatory border just inside the choroid. At times masses of bacteria could be seen in the necrotic area. Many necrotic heterophils are found in the necrotic area, particularly near the margin. The border was at first composed of heterophils and mononuclear phagocytes, but

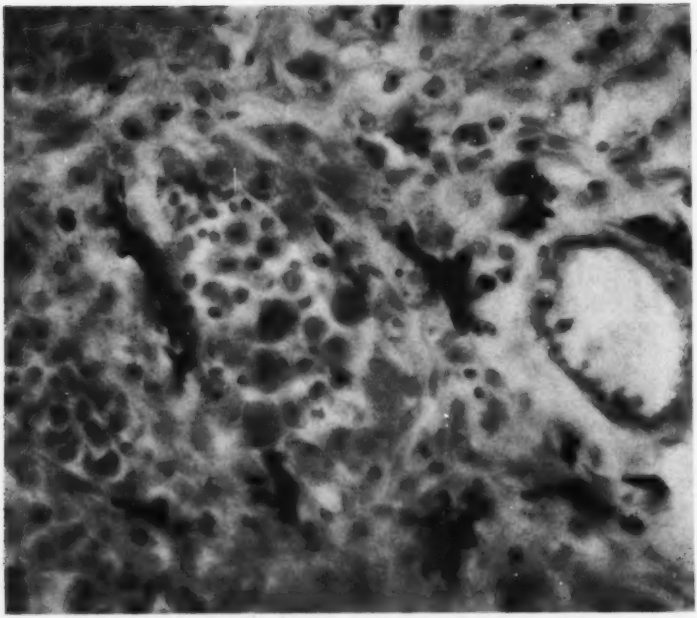

Fig. 3 An early lesion of the choroid. Heterophils and mononuclears are both present and a few heterophils have been engulfed by mononuclear phagocytes. × 600.

later it became lined with giant cells. Occasionally, similar large necrotic masses were found in the aqueous.

After the infection was controlled, healing by organization occurred. The choroid remained greatly thickened and congested, and there were fibroblasts as well as nests of mononuclear phagocytes between the blood vessels. The exudate in the anterior and posterior chambers also healed by organization, and the over-all size of the eye was somewhat reduced.

The eye lesions resulting from air sac infection with *E. coli* alone or with egg-propagated PPLO could not be differentiated.

CONCLUSIONS

An experimentally produced panophthalmitis in association with severe *E. coli* bacteremia is described. Infection was

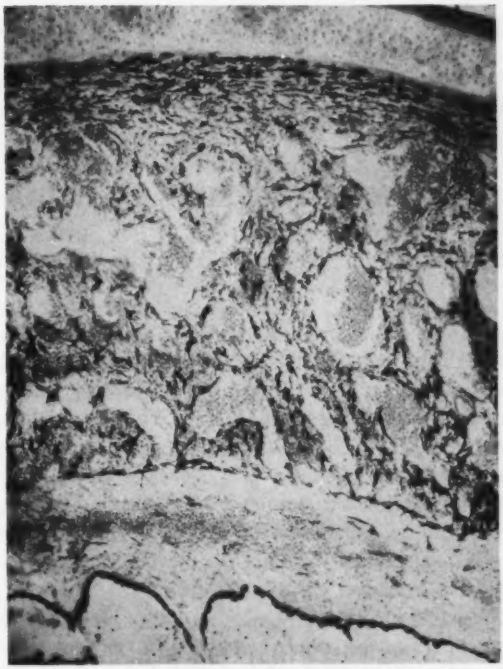

Fig. 4. More developed lesion of the choroid with great hyperemia and mononuclear infiltration. There is hyperemia and mononuclear infiltration of the degenerate retina. × 100.

produced by inoculating broth cultures of certain types of *E. coli* alone or with egg-propagated PPLO into the lesser abdominal air sacs of chickens. The chief symptoms were blindness associated with hypopyon. The microscopic lesions consisted of an infiltration of the entire uveal tract by mononuclear phagocytes and heterophils. This was accompanied by extensive edema and hyperemia of the choroid and iris. A heterophil-mononuclear exudate was present in the anterior chamber, and granulomas sometimes developed in the choroid, ciliary body, viterous humor, or aqueous humor. Similar infections have been noted in field flocks.

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STUDIES ON ERYSIPELAS IN TURKEYS*

By CLYDE I. BOYER, JR., and J. A. BROWN

Department of Pathology and Bacteriology, New York State Veterinary College, Ithaca, New York

ERYSIPELAS was first reported in New York State turkeys in 1938 by Van Roekel.[15] Since then numerous cases of erysipelas have been found in New York[5,11] and the disease is now recognized as a considerable economic problem in adult turkey flocks.

In experimental work on turkey erysipelas some workers have had difficulty in maintaining virulence of *Erysipelothrix rhusiopathiae* cultures and obtaining a consistent and high mortality in inoculated turkeys. Adler and Spencer[1], Dickinson *et al.*[6], and Moynihan and Stovell[10] reported that an irregular mortality resulted when susceptible turkeys were challenged by other than the intravenous route. Adler and Spencer[1] also mentioned that it was difficult to maintain virulence of *E. rhusiopathiae* cultures that were lyophilized.

Peterson and Hymas[12] reported good mortality when the intramuscular route was used in non-immune turkeys. The percutaneous (skin scarification) route as originally described by Fortner and Dinter[7] was found to be the only way to reproduce erysipelas in swine consistently.

Some work has been done on the efficacy of vaccines for protection of turkeys. Dickinson, *et al.*,[6] reported a variable degree of protection to experimental challenge of vaccinated field flocks. Jerstad and Johns[9] reported that vaccinated flocks

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showed a high degree of protection against natural outbreaks even when vaccination was delayed until deaths from erysipelas occurred. Moynihan and Stovell[19] have reported some promising results with oral antibiotic therapy on small numbers of turkeys. Ullberg[14] obtained high activity *in vitro* against *E. rhusiopathiae* with penicillin and fairly high activity against the same organism with terramycin and aureomycin. He stated that these antibiotics may prove of value if put into the feed but that there was little reason to assume that any of these substances would prove to be more effective than penicillin in clinical use.

Our efforts were designed to find the possible ways that transmission might occur and to investigate further the possibilities of vaccination and antibiotic therapy for controlling this disease. In order to carry on experimental studies we tried to find a way to keep a strain of the organism virulent and to produce consistently a high mortality in inoculated turkeys. Since intravenous inoculations pose practical difficulties and since this method, according to some workers,[6] may produce too severe a challenge for evaluating some experimental results, we decided to try intramuscular and skin scarification methods with two types of inoculums.

MATERIALS AND METHODS

The same strain of *E. rhusiopathiae* was used in all the experiments reported in this paper. It was isolated from a field outbreak in turkeys in October 1953 and kept viable and virulent by monthly passage in poults. The livers from these poults were stored in sterile petri dishes at refrigerator temperatures. When needed, 24 hour chicken infusion broth cultures were prepared from these livers.

In all our experiments, *E. rhusiopathiae* was isolated from at least 20 percent of birds dead or dying from the infection. In many instances, particularly when small numbers of birds were involved, all dead turkeys were cultured. The criteria used for identification of *E. rhusiopathiae* were those given by Bergey's Manual of Determinative Bacteriology[4].

The inoculations via the foot pad and skin were done by scarifying about a one-quarter to one-half inch area of the

skin of the foot pad or breast with sand or emery paper until a small amount of serum or blood oozed out. About 0.03 ml of a 24 hour broth culture was rubbed into this area with a sterile swab. The feather follicle inoculation consisted of pulling out 6 to 8 small feathers along the side of the breast and rubbing about 0.03 ml of a broth culture into the follicles. Infection via the snood was done by cutting off the end of the snood with sterile scissors and rubbing a broth culture into the cut area. The nasal, tracheal and conjunctival methods consisted of dropping 0.03 ml of a broth culture into the chosen area with a sterile syringe and needle.

The inhalation method was done by subjecting the bird for two minutes to an aerosol mist from a De Vilbiss #40 nebulizer of a 24 hour broth culture of *E. rhusiopathiae*. Either the upper beak or the entire head of the bird was introduced into one end of an air-tight glass cylinder through a rubber dam and the aerosol sprayed from the other end. The conjunctival spray was one done by placing one end of a glass tube six inches long and five-eights of an inch in diameter firmly over the eye. Spray from a De Vilbiss nebulizer was directed for two minutes through a one-hole rubber stopper in the other end of the eye exposed to an aerosol mist of one-half to one-quarter ml of a culture.

RESULTS

Table I shows the results when two different types of inoculums were used by two different routes of infection. Both types of inoculums and both routes of inoculation gave consistently high mortality with the same strain of organism in young and adult turkeys.

Table II shows the results when a 24-hour, broth culture was introduced by different routes into turkeys of various ages.

In selecting birds for use in experimental work, the question arose as to what age and sex of bird should be used. Our experimental infections via intramuscular inoculation of broth cultures showed that a consistently high mortality resulted with both young and adult turkeys as seen in table III.

TABLE I

Mortality from liver suspension and broth culture inoculums of *E. rhusiopathiae* by intramuscular and breast skin scarification methods in turkeys 3 to 30 weeks of age.

Total No. of exp. done	Inoculum	Route of Infection	Age	No. died/ no. inoc.	% Mortality Average	% Mortality Median	Remarks
1	Liver	IM	8 wks.	23/24	96		All birds in this trial were inoculated at the same time and with the same two inoculums.
1	Broth Cult.	IM	8 wks.	23/24	96		
1	Liver	BSF	3 wks.	12/16	80		
1	Broth Cult.	BSF	3 wks.	15/15	100		
32	Liver	IM	3 to 30	145/177	82	83	The birds in these trials were inoculated at different times and with different passages of the same strain of organism.
23	Broth Cult.	IM	3 to 30	153/193	79	85	
10	Liver	BSF	3 to 30	45/55	82	100	
14	Broth Cult.	BSF	3 to 30	69/81	85	100	

0.1 ml in breast of above inoculums was used in all IM experiments.
IM = Intramuscular
BSF = Breast skin scarification

TABLE II

Mortality after inoculating a 24 hour broth culture of *E. rhusiopathiae* into turkeys 3 to 30 weeks of age by various routes.

Inoculation Route	Total no. exp. done	No. dead/ no. inoc.	% Mortality Average	% Mortality Median
Intravenous 0.1 ml	5	32/32	100	—
Subcutaneous 0.1 ml	1	11/12	92	—
Foot pad scarification 0.03 ml	1	7/8	88	—
Breast skin scarification 0.03 ml	14	81/93	87	100
Intramuscular 0.1 ml	28	239/308	77	86
Feather follicle 0.03 ml	3	12/16	76	50
Clipped snood 0.03 ml	2	15/20	75	—
Spray—conjunctiva	1	0/10	—	—
Spray—nares	1	3/10	30	—
Spray—entire head	2	5/20	25	—
Conjunctival 0.03 ml	2	4/27	15	—
Tracheal 0.03 ml	2	4/31	13	—
Nasal 0.03 ml	1	0/10	—	—
Per orum 0.5 ml	8	0/25	—	—

There is a feeling on the part of some workers[8] that male turkeys are more susceptible to erysipelas than females. Table III has data from three experiments on the mortality in mixed sex groups of 5 to 7-week poults. There was no difference in

the mortality of either sex in this age group. Although the differences in mortality between the sexes in the five experiments in groups of adult males and females were greater, we feel that these results could have been due to chance. The X^2 test was not significant.

TABLE III

Effect of age and sex on mortality after intramuscular inoculation of experimental turkeys with 0.1 ml 24 hour broth culture of *E. rhusiopathiae*

Total number Exps. done	Age	Sex	Died/Inoc.	% Mortality
23	2- 8 wks.	Mixed	153/193	79
5	20-30 wks.	Mixed	86/115	75
1	5 wks.	Male	13/14	93
	5 wks.	Female	11/12	91
1	5 wks.	Male	9/10	90
	5 wks.	Female	9/10	90
1	7 wks.	Male	7/8	87
	7 wks.	Female	11/12	91
Total Poult	5-7 wks.	Male	29/32	90
Mortality	5-7 wks.	Female	31/34	91
1	23 wks.	Male	9/14	60
	23 wks.	Female	12/12	100
1	24 wks.	Male	9/12	75
	24 wks.	Female	9/12	75
1	29 wks.	Male	12/12	100
	29 wks.	Female	7/12	58
1	30 wks.	Male	6/8	75
	30 wks.	Female	9/15	60
1	31 wks.	Male	5/5	100
	31 wks.	Female	5/12	42
Total adult	23-31 wks.	Male	41/51	80
Mortality	23-31 wks.	Female	42/63	67

Results on the challenge of turkeys vaccinated with Duovax (erysipelas bacterin) are in table IV. There is evidence of a variable degree of protection from this bacterin up to 20 weeks post vaccination. For some unexplainable reason the birds challenged at 21 weeks had no protection when compared with non-immune controls. An important consideration in these trials, however, was the fact that 50 percent or more of the vaccinated birds in each experiment became visibly sick.

Preliminary pilot trials with infected poults given 600 grams of bacitracin per ton in the feed or 500 mgs per gal-

TABLE IV

Results of Experimental Challenge of Turkeys Vaccinated at 8-12 weeks with a Commercial Erysipelas Bacterin (Duovax-Lederle).

Trial No.	Breed & No.	Age in wks.	Sex	Treatment	Time between Vaccination & Challenge +	% Highest Morbidity	Mortality Sex	Mortality %	Diff. %
1	WH	23	14 M 11 F	Vaccinated	10½ wks.	60	7M 4F	44.0	
	51								36.8*
			14 M 12 F	Non-vaccinated	10½ wks.	100	9M 12F	80.8	
2.	Emp	25	25 M	Vaccinated	15 wks.	50	7M	28.0	
	50		25 M	Non-vaccinated	15 wks.	100	21M	84.0	56.0*
3.	BBB	29	12 M 12 F	Vaccinated	19½ wks.	55	7M 3F	41.7	
	48								37.5*
			12 M 12 F	Non-vaccinated	19½ wks.	100	12M 7F	79.2	
4.	Emp	30	8 M 14 F	Vaccinated	20 wks.	50	3M 2F	22.7	
	45								42.5*
			8 M 15 F	Non-vaccinated	20 wks.	100	6M 9F	65.2	
5.	BBB	31	14 M 11 F	Vaccinated	21 wks.	80	10M 6F	64.0	
	42								(-5.2)
			5 M 12 F	Non-vaccinated	21 wks.	87	5M 5F	58.8	

+ 0.1 ml 24 hour Broth Culture — IM Breast
WH = White Holland
Emp = Empire White
BBB = Broad Breasted Bronze

M = Male
F = Female
* Statistically significant difference in favor of vaccinates

lon in the water (as "aquatracin"), 150 grams of furazolidone (NF 180) per ton in the feed, 200 grams of terramycin per ton in the feed or 500 mgs per gallon in the water, and 2000 mgs of streptomycin per gallon in the water gave discouraging results. Table V shows the results of an experiment with limited numbers of young and adult turkeys fed aureomycin and penicillin in the ration. The birds in these trials were medicated continuously during the ten day course of the experiment. The antibiotic intake for the first four days is indicative of the trend of drug intake for the rest of the medication period. Most of the mortality occurred during the same 4 day period. The best results with the older birds were obtained in feed with 600 grams of aureomycin per ton or 325 grams or more of procaine penicillin per ton. One hundred percent of the aureomycin medicated birds got sick compared with 30 percent of the penicillin fed groups. Despite this, two birds died in the penicillin group as against one in the aureomycin group. Drug intake calculated as mgs per pound of body weight was much greater in young poults than in older birds. This probably explains why, in most of our pilot trials, the mortality was consistently lower in poults than in the older birds at the same drug levels. There was no mortality in 5-week, aureomycin-medicated poults. The mortality (40 percent) in the young poults on penicillin was surprising and not in agreement with previous results in pilot trials.

DISCUSSION

Many theories have been advanced about the way in which natural outbreaks of erysipelas occur and transmission takes place. Some workers[13,3] state that infection in swine and turkeys may occur via external wounds or through breaks in the mucous membranes. Our experimental results with exposure via the foot pad and breast skin scarifications tend to support this view. Theories have also been advanced that natural infections may take place through the respiratory tract in turkeys[2], and in swine[13] by ingestion of virulent organisms. We did get a low mortality from infections via the respiratory tract and the conjunctiva. In fact, the results from these last two inoculation routes are similar to the per-

TABLE V

Effect of antibiotic intake on morbidity and mortality in 29-week and 5-week-old turkeys inoculated intramuscularly with 0.1 ml of 24 hour broth culture of *E. rhusiopathiae*. Each treatment group was made up of 10 birds averaging 24.9 and 1.29 lbs. respectively

AGE	Day of Infection	Aureomycin* 325 gm/ton	Aureomycin 600 gm/ton	Penicillin** 325 gm/ton	Penicillin 600 gm/ton	Penicillin 800 gm/ton	Infected controls non-medicated
29 weeks			Antibiotic intake—mg per lb. body weight				
	1	10.57	19.72	10.48	22.62	30.41	
	2	5.75	13.03	8.85	19.72	23.74	
	3	3.04	14.58	7.73	19.36	25.69	
	4	1.49	9.44	7.40	17.42	25.37	
			Morbidity peak day				
		100%	100%	30%	20%	0%	100%
			Mortality ten days				
		50%	10%	20%	20%	0%	80%

AGE	Day of Infection	Aureomycin* 325 gm/ton	Penicillin** 325 gm/ton	Infected controls non-medicated
5 weeks			Antibiotic intake—mg per lb. body weight	
	1	33.83	31.79	
	2	26.37	23.90	
	3	25.12	28.99	
	4	25.12	35.98	
			Morbidity peak day	
		60%	70%	100%
			Mortality ten days	
		0%	40%	80%

* Aureomycin — T. F. (American Cyanamid Co.)
** Penline — 200 — Procaine penicillin (Commercial Solvents Corp.)
There was no morbidity or mortality in negative controls (uninfected, non-medicated) that were included with each age group.

centages of mortality often experienced in some natural field outbreaks in turkeys. Our attempts to infect turkeys by the oral route failed.

Our results with vaccinated turkeys indicated a reduction of mortality following challenge up to 20 weeks post-vaccination. However, considerable morbidity in the vaccinates was observed and this situation in the field would undoubtedly prompt the turkey grower to inject those birds individually with penicillin. In such a case, successful medication with antibiotics in the feed would certainly be a cost saving solution. From a practical and economic standpoint, our results with procaine penicillin in the feed are promising and further studies seem justifiable.

SUMMARY

Experimental infections with *E. rhusiopathiae* were done with a total of 732 turkeys to determine the best route of inoculation, efficacy of vaccination, and value of antibiotic medication.

The virulence of a strain of *E. rhusiopathiae* has been maintained since October 1953 by frequent passage by intramuscular inoculation of turkeys. Infected livers stored in a refrigerator were used for inoculums.

Experimental transmission and mortality resulted from exposures of turkeys by a number of different inoculation routes. The following routes listed in order of highest mortality (at least 75 percent) were: intravenous, subcutaneous, foot pad scarification, breast skin scarification, intramuscular, and feather follicle.

Young poults and adult turkeys were about equally susceptible to experimentally induced erysipelas.

Sex of young poults and adult turkeys did not have any effect on susceptibility and mortality in experimental erysipelas.

In four trials, turkeys vaccinated with "Duovax" bacterin suffered from 36 to 56 percent fewer deaths than non-vaccinated controls, when challenged up to 20 weeks with virulent cultures. One trial resulted in no protection.

Bacitracin up to 600 grams per ton in the feed or 500 mgs. per gallon in the water (as "aquatracin"), furazolidone (NF 180) at 150 grams per ton in the feed, terramycin at 200 grams per ton in the feed or 500 mgs per gallon in the water, and streptomycin at 2000 mgs per gallon in the water gave inadequate protection to young poults in pilot trials. When turkeys 29 weeks of age were fed 325 grams or 600 grams of aureomycin per ton and 325, 600 or 800 grams of procaine penicillin per ton, the mortality from experimentally induced erysipelas was reduced by 30, 70, 60, 60 and 100 percent respectively. The morbidity was much higher in the aureomycin fed birds.

Five-week-old turkeys fed 325 grams aureomycin per ton or 325 grams penicillin per ton had 100 percent and 40 percent fewer deaths from experimental erysipelas than the controls.

ACKNOWLEDGEMENTS

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CASE REPORT——A PARATYPHOID OUTBREAK IN A POULTRY BREEDING FLOCK

By C. I. ANGSTROM

N. Y. State Veterinary College, Regional Poultry Disease Laboratory, Kingston, N. Y.

ON November 2nd, 1955 four New Hampshires and five Single Comb White Leghorns were presented to the laboratory for examination. These birds were from a prospective breeding flock of 1,000 New Hampshires and 4,000 White Leghorns. They had reacted to the official whole blood plate test for pullorum disease. The birds had been hatched on the same premises from a pullorum clean breeding flock.

At the laboratory the birds were tested again with the standard, polyvalent and variant pullorum antigens by the whole blood plate method. The results of the retests, the lesions and results of bacteriological examination are shown in table I.

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TABLE I

Results of whole blood plate test with pullorum antigens anl post mortem examination of reactors

Birds		Whole Blood Plate Test Antigen				Bacteriological Cultures	
#	Breed	Standard	Poly.	Variant	Lesions	Blend of Viscera	Gut
1	N H	3+	3+	3+	—	*S. give*	—
2	N H	2+	PP	—	—	—	—
3	N H	1+	PP	PP	—	—	—
4	N H	3+	2+	1+	—	—	—
5	W L	PP	—	—	—	*S. typhimurium*	*S. typhimurium*
6	W L	3+	2+	1+	*	—	—
7	W L	1+	PP	—	—	*S. typhimurium*	*S. typhimurium*
8	W L	PP	—	—	—	—	—
9	W L	1+	PP	—	**	*S. typhimurium*	*S. typhimurium*

PP = pinpoint or slight agglutination
1+ = some agglutination
2+ = moderate agglutination
3+ = complete agglutination
* = yolks in peritoneal cavity
** = peritonitis

A retest of the flock a month later revealed 33 reactors by the time half the birds in the flock were retested. Agglutination titers of these birds were lower than those of the previous group. On post mortem examination, two birds showed gross lesions consisting of a peritonitis in one bird and plaques in the lower bowel of the other. *S. typhimurium* was isolated from 13 of the 33 birds submitted. Neither bird with gross lesions yielded the organism, and as in the first group, there was a disappointing lack of correlation between the agglutination titers and the isolation of *Salmonellas.*

The flock was liquidated as recommended when the presence of *S. typhimurium* was confirmed in the second group of birds. At no time did this flock show any clinical indications of the *Salmonella* infection. Both livability and egg production were excellent up to the time of liquidation.

This case is reported as an instance in which paratyphoid infection was responsible for the elimination of a prospective breeding flock. The infection was detected by the official whole blood plate test for pullorum disease. The serological identification of *Salmonella typhimurium* and *Salmonella give* was made by Dr. D. W. Bruner at the N. Y. S. Veterinary College.

THE ISOLATION OF A VIRAL AGENT FROM CHICKENS SHOWING RESPIRATORY DISTRESS*

By M. S. COVER and W. J. BENTON

University of Delaware Agricultural Experiment Station
Newark, Delaware

IN 1954, Crawley and Fahey[1], reported the isolation of a new viral agent from chickens showing clinical evidence of chronic respiratory disease (CRD). Since that time, we have attempted to make similar isolations from affected birds in Delaware. The following report includes part of the work involved in isolating and investigating the properties of one virus isolated (virus 146).

The virus 146 in question was isolated from birds out of a flock of 12,500 broiler chickens 6½ weeks of age. They were reported to be suffering from a respiratory distress for 1 week prior to our visit. The history of this flock was reported as follows: Newcastle vaccination was done by spray method, B-1 strain at 10 days and 4½ weeks of age. There was no complaint of previous trouble in the flock prior to the current difficulty. The respiratory disease began at about 6 weeks of age. The first symptoms were coughing, sneezing and moisture about the anterior nares and eyes. There was a reduction in the amount of feed consumed. During the next few days, the amount and intensity of the respiratory symptoms increased, viz., moist râles and increased coughing. There was no evidence of gaping or gasping for breath.

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At necropsy these birds showed the typical pathological changes of CRD. These were thickened air sacs which contained thick, yellow exudate, perihepatitis and pericarditis, etc. Suitable specimens were brought to the laboratory for additional study. Our report at necropsy contained, in addition to the changes above noted, sinusitis, rhinitis and laryngitis, all of which were characterized by congestion and mucous exudate. In addition, the mucous surface of the larynx showed small petechial hemorrhages. There was a fluid mucus present in the trachea, but no evidence of congestion or hemorrhage of any type. The bronchi and lungs were without gross pathologic change.

Material obtained by a tracheal scraping was suspended in sterile nutrient broth. This suspension was then injected into the chorio-allantoic sac (CAS) of 7-day and 10-day embryonated chicken eggs. In the first passage, the material was not fortified with antibiotics and the embryos died during the first 48 hours. Following this experience, the inoculum was fortified with 20,000 units of penicillin and 10 milligrams of streptomycin per ml. The embryo mortality and pathologic changes occurring in this initial and subsequent passages are shown in table I.

This virus has been passed for 21 successive times in embryonated chicken eggs inoculated into the CAS in the quantity of 0.2 ml using chorio-allantoic fluid (CAF) harvested from the previous passage. It has not changed its activity throughout these passages. Two interesting lesions were noted in the embryos which died following inoculation. These were (1) the CAF became thick and grey to white in color, (2) the heart showed definite myocardial involvement in that the surface contour was marred by irregularities. As the virus was handled in the laboratory, attempts were made from time to time to investigate its biological properties. The results of filtration experiments made with Seitz filters are shown in table II. The virus passed through the 2.0 micron pad but was held by the 0.1 mm. pore size filter. Harvested chorio-allantoic fluid produced embryo mortality when incu-

TABLE I

Results of passages in embryonated chicken eggs of virus 146

Source of material	Treatment	Route of inoculation	Mortality/ no. inoculated	Lesions in embryonated chicken eggs
1A Original tracheal scraping	none	CAS	5/5	Bacterial contamination
1B Original tracheal scraping	none	CAM	5/5	Bacterial contamination
1C Original tracheal scraping	none	YS	5/5	Bacterial contamination
2A Original tracheal scraping	Penicillin 20,000 units, Streptomycin 10 mgm.	CAS	2/6	Necrosis liver-plaques CAM
2B Original tracheal scraping	Penicillin 20,000 units, Streptomycin 10 mgm.	CAM	1/5	NVL
2C Original tracheal scraping	Penicillin 20,000 units, Streptomycin 10 mgm.	YS	2/5	Some bacterial contamination
3A Harvest CA fluid 2A	none	CAS	4/6	Necrosis liver-thick yolk
3B Harvest CA fluid 2B	none		0/5	NVL
4A Harvest CA fluid 3A	none	CAS	3/4	Necrosis liver-plaques CAM
5A Harvest CA	none	CAS	6/6	Same as 4A

CAS = chorio-allantoic sac
CAM = chorio-allantoic membrane
YS = yolk sac
NVL = no visible lesions

bated for only 5 hours at 37½C. The addition of 40,000 units of penicillin or 40 milligrams of streptomycin, or both, per ml of culture did not reduce the pathogenicity of the virus.

The inoculation of this virus into birds gave the most interesting results. The original material (tracheal scraping) was inoculated into two groups of 10 birds each. The birds were 6 weeks of age and each received 0.2 ml of the material intratracheally. These birds were held in isolation for 21 days and did not show any signs nor symptoms of respiratory distress during that period. At necropsy a mild rhinitis, sinusitis and laryngitis was found. There was a small amount of very fluid mucus in the larynx and trachea. Since this time, birds of various ages have been inoculated with this virus without producing mortality. Birds 3 weeks, 6 weeks, 8 weeks and 1 year of age have been inoculated intranasally or intratracheally with infective chorio-allantoic fluid. Usually neither symptoms nor mortality occurred following the administration of the virus, but in some cases mild respiratory symptoms of sneezing and coughing occurred after inoculation but soon disappeared. The virus has been reisolated from these birds when tracheal scrapings were made on the 7th day following inoculation.

Since the virus was growing well in embryonated chicken eggs and upon titration gave a rather constant mortality pattern and titre (10^3 to 10^4), it was decided that the serum neutralization test could be of value in immunization experiments.

TABLE II

Results of filtration experiments with virus

Pore-size of filter	Number dead/ Number inoculated	Number dead/ Number inoculated
	Test #1	Test #2
5.0 microns	5/5	5/5
2.0 microns	2/5	2/5
0.1 microns	0/5	0/5
Not filtered	5/5	5/5

In this experiment, three birds were exposed by placing 0.2 ml of harvested CAF (6th embryo passage) intratracheally. Three additional birds were held in isolation as a control. The serum neutralization titre to the 146 virus and to infectious bronchitis were ascertained at pre-inoculation, 10 days post-inoculation and, 30 days post-inoculation. The serum plate agglutination test for the pleuropneumonia-like organism (PPLO) of CRD was also performed at the same intervals. The 146 virus produced a significant serological response in 30 days and a response was detectable on the 10th day post-inoculation (table III).

TABLE III

Serological response of 6½-week-old birds inoculated intratracheally with 0.2 ml of virus 146

		Pre-inoculation			10 days post-inoculation			30 days post-inoculation		
		SN 146	SN Bron.	PPLO agglut.	SN 146	SN Bron.	PPLO agglut.	SN 146	SN Bron.	PPLO agglut.
Birds exposed	1	0	2.12	N	1.22	*	N	2.86	1.05	N
	2	0	2.17	N	1.31	*	N	4.16	2.32	N
	3	0	2.63	N	0.80	*	N	4.16	4.21	N
Birds not exposed	1	0	1.24	N	0	*	N	0	4.10	N
	2	0	2.24	N	0	*	N	0.91	2.71	N
	3	0	4.00	N	0.02	*	N	0	*	N

* = not done

The serum neutralization titre of 12-week-old birds inoculated 30 days previously with 146 virus as compared to serum from birds immunized with laryngotracheitis vaccine by the usual method (also 30 days previously) was also ascertained. The serum from the birds receiving the 146 virus had a positive neutralizing titre against the 146 virus but a much lower antibody titre against laryngotracheitis virus. The serum of birds vaccinated with laryngotracheitis vaccine had no antibodies against the 146 virus (table IV).

The next experiment, although designed along the same pattern, was expanded to include a study of the time required for serological response as well as the development of pathologic changes in the respiratory tract. In this test, 28 birds 6½ weeks of age were placed in a small colony house. The birds received 0.2 ml of virus 146 intratracheally. The hem-

TABLE IV

Serological response of birds immunized against virus 146 and laryngotracheitis when exposed to challenge with these viruses

	Titre of virus alone	Serum titre of birds inoculated with virus 146	Serum titre of birds hyper-immunized with laryngotracheitis
Virus 146	4.00	>4.00	0
Virus laryngotracheitis	4.37	2.87	2.03

agglutination inhibition titre for Newcastle disease, serum plate agglutination test for the PPLO of CRD, and the serum neutralization test for infectious bronchitis and virus 146 were determined before exposure to virus 146 as well as on the 10th, 15th, 20th, 25th and 30th days after exposure. At each time interval, some birds were destroyed and examined for gross lesions of the respiratory tract. Five susceptible birds were placed in the colony house as contacts on the 12th day and three more on the 20th day after exposure. These birds remained in the pen for 30 days. The results of this experiment are shown in table V.

It is to be noted that the serological response to virus 146 in inoculated birds was detectable at the first test, 10 days after exposure and reached its height on the 25th day. None of the serological tests for other antibodies were positive in these birds throughout the experiment. The gross lesions were confined to a mild aerosacculitis and tracheitis.

The results of the serological tests with the contact chickens were confusing. None of the susceptible, introduced birds had SN antibodies to virus 146. However, five out of eight birds did yield positive SN titers to infectious bronchitis. An adequate explanation for this cannot be given since bronchitis antibody titers were not found in any serum samples (pooled) from the other birds in this pen.

A cross immunization test was also made with virus 146 and laryngotracheitis virus (vaccine strain). Twenty birds, 8 weeks of age, were vaccinated in the vent with laryngotracheitis vaccine and twenty additional birds were vaccinated (vent) with the virus 146. All birds showed a reaction in the

TABLE V

Serological response and development of pathological changes in birds inoculated with virus 146 and in contact control birds.

Bird nos.	No. days before or after infection	Virus 146 SN	Gross Pathology
1-5	—7	0	None
6	+10	1.98	Rhinitis, tracheitis
7		0	air sacs negative
8		1.13	
9	+15	1.82	Aerosacculitis.
10		5.24	Mucous tracheal exudate
11		3.24	without hemorrhage
12		1.92	some air sacs with cheesy exudate
13-15	+20	4.49	Same as above
16		1.99	
17-19	+25	4.15	Same as above
20		2.82	
21		1.53	
22		2.34	
23		0.93	
24	+30	2.56	Lesions less severe
25		0.78	
26		1.50	
27		1.81	
28		1.80	

vent on the 5th day. Revaccination of 10 birds with laryngotracheitis vaccine (vent) and 10 with the virus 146 from each of the above groups 30 days later produced no reaction in the vent.

In the next experiment 16 chicks, 3 weeks of age, were inoculated by intranasal instillation with harvested chorioallantoic fluid. Mild respiratory symptoms were present in the 4th, 5th and 6th days following vaccination. Twenty-five days later eight were challenged by intranasal instillation and eight by vaccination into the vent (laryngotracheitis virus). None showed any reaction to the challenge. Three control birds vaccinated with the virus 146 in the vent showed definite takes on the 5th day.

In another experiment ten New Hampshire chickens 8 weeks of age were inoculated with one drop of the virus 146

intranasally and 0.1 ml intratracheally. Ten other birds were held in the same pens as the contact controls. In addition, 10 birds were held in isolation and were not inoculated with the virus.

Mild respiratory symptoms, such as sneezing and coughing, were noted in the inoculated group two days after exposure. The symptoms persisted for 5 to 7 days. Twenty-six days later all birds were vaccinated with laryngotracheitis vaccine obtained from a commercial laboratory. The vaccination was accomplished by brushing the vaccine into the mucosa of the vent. On the 4th and 5th day after vaccination, all 10 birds inoculated with the virus 146 were negative for a vaccination take. All uninoculated control birds were positive, and eight of the ten contacts were negative with two questionable reactions.

CONCLUSIONS

We believe our virus 146 to be that of laryngotracheitis. It may well be that some of its characteristics differ from those usually attributed to laryngotracheitis. At certain stages of our research, the results indicated that the virus had some properties which would indicate at least some similarity to the virus of Crawley. Continued work, however, has indicated that this virus is more closely associated with that of laryngotracheitis.

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A MODIFIED CHICKEN EMBRYO INOCULATION TECHNIQUE FOR THE ISOLATION OF VIRUSES FROM THE RESPIRATORY TRACT OF CHICKENS*†

By JULIUS FABRICANT

Department of Pathology and Bacteriology
New York State Veterinary College
Cornell University, Ithaca, New York

IN January 1956 a large series of virus isolation studies was started in an attempt to determine the incidence of avian infections with Fahey and Crawley[4] virus in New York State. One phase of these studies consisted of virus isolation attempts with at least three serial passages by the allantoic sac route (AA) and three serial passages on the chorio-allantoic membrane. The chorio-allantoic technique used was one devised by Dr. John Gorham of Washington State College and modified somewhat by Dr. J. H. Gillespie of the Veterinary Virus Research Institute of the New York State Veterinary College. This technique (designated as the CCA method) was tested with several known strains of Fahey and Crawley virus prior to the isolation studies and was found to be the best technique for the cultivation of this agent when compared with the allantoic sac or yolk sac routes of inoculation.

Although no agents resembling Fahey and Crawley virus were isolated in this study, it soon became apparent that this

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CCA technique was an improvement over our previous routine methods of isolating respiratory disease viruses in fowl. Isolations of Newcastle disease (including B_1 strain), infectious bronchitis, fowl pox and infectious laryngotracheitis viruses were made by this modified CCA method.

It has long been known that the CA technique was preferable for the isolation, identification and cultivation of fowl pox[6] and laryngotracheitis[1]. In the case of Newcastle virus, the route of inoculation was usually of little importance. However, for many years as a result of the work of Delaplane[2] and Fabricant[3], the accepted method for the isolation of infectious bronchitis virus has been the allantoic sac technique. The early results of this study indicated that this CCA technique gave as good or better results than the routine AA technique when run in parallel from aliquot samples of the same suspension of respiratory tract tissues (ground lung or tracheal swab). Accordingly, a large series of comparative isolation attempts were made from respiratory tract material which was suspected of containing infectious bronchitis virus.

MATERIALS AND METHODS

The allantoic sac inoculation technique used in this experiment was previously described in detail[3].

The modified chorio-allantoic technique, was done as follows: The chick embryos were candled for viability at 9 to 10 days of age. A small hole was drilled on the top of the egg over the center of the air sac, cutting through the shell but leaving the shell membrane intact. The inoculation was made with a one ml tuberculin syringe with a 1½ inch, 22 gauge needle. The egg was held upright in a tray or box with the drilled hole facing directly upward. The needle held vertically was inserted into the hole and pushed gently through the shell membrane to a depth of one-quarter inch. The inoculum was then dropped on the air sac membrane. Then the needle was pushed directly downward through the pool of inoculum toward the center of the egg to a depth of 1¼ inches. This technique carried virus on to the CA membrane and also into the allantoic sac. The eggs were then sealed and incubated.

Virus Isolation Technique.

Tracheas and lungs were collected from cases of respiratory diseases submitted to the poultry diagnostic laboratories associated with the New York State Veterinary College. These tissues were stored at —20C until virus isolations were attempted. The tissues were thawed and suspensions of tracheal exudate and ground lung were made in nutrient broth (Difco) pH 6.8 containing 10,000 units of penicillin and 5 mg. of streptomycin per ml for bacteriostatic purposes. These suspensions were held at room temperature for one hour before their inoculation into chicken embryos.

In the course of each isolation six embryos were injected with 0.2 ml of inoculum by each route (AA and CCA) from the same tissue suspension. The embryos were candled daily. If no deaths occurred by the time the embryos were 15 days of age, an embryo was selected by candling representative of each route. These embryos were examined and embryonic fluids or membranes harvested for serial passage. If none of the embryos appeared abnormal at this candling, one was selected at random. The remaining embryos were candled daily until 19 days of age, at which time all were broken open and examined for lesions. These procedures were continued through three serial passages when necessary. Six embryos were used per passage. The viruses encountered in these embryos were identified by the usual accepted criteria.[1,3,5]

RESULTS

In a series of over 150 comparative virus isolation attempts, infectious bronchitis virus was isolated 58 times. The comparative efficacy of the two methods is summarized in table I.

TABLE I

A comparison of the efficiency of two embryo inoculation techniques in the isolation of infectious bronchitis viruses

	Number
Virus isolated in fewer passages by AA route	2
Virus isolated in fewer passages by CCA route **	14
Virus isolated in same number of passages by either route	13
Virus isolated by AA route but not by CCA route	3
Virus isolated by CCA route but not by AA route	26

** Average CCA isolation 1.33 passages sooner than corresponding AA isolation.

It can be seen by examining the data in table I that the advantage was to the AA route in 5 of 58 isolations. The advantage was to the CCA route in 40 of 58 isolations. Both routes were equally effective in 13 of 58 isolations. When we summarize these figures we find that the AA route was as good or better than the CCA route 18 times, and that the CCA route was as good or better than the AA route 53 times. Mainland's[5] table of Binomial Confidence Limits indicates that these data are highly significant. The odds are 100 to 1 that the CCA method will be better from 71.6 to 97.5 percent of the time.

Data from the same experiment is arranged in table II to compare the number of embryo passages needed to make positive isolations of infectious bronchitis by the two routes.

TABLE II

The number of embryo passages needed to make positive isolations of infectious bronchitis by AA and CCA routes

	1st passage	2nd passage	3rd passage	4th passage	Total isolations
Allantoic route AA)	10 32%	12 39%	7 22%	2 6%	31 Isolations
Chorio Allantoic route (CCA)	30 56%	16 30%	7 13%	1 2%	54 Isolations

It can be seen by an examination of these two tables that use of the CCA method resulted in both an increase in the number of isolations and in the speed with which these isolations could be identified. Although the CCA route more often gave a positive diagnosis in the first and second passage than the allantoic route, there were still sufficient isolations on the third passage to make it necessary to continue blind passages at least to that point.

The increased effectiveness of this CCA technique for the isolation of infectious bronchitis virus together with its ability to detect fowl pox, laryngotracheitis and Newcastle viruses suggest that this is a simpler and more effective method for the routine detection of these agents in avian respiratory infections.

ACKNOWLEDGEMENTS

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SUMMARY

A simple chicken embryo inoculation technique for the isolation of viruses from avian respiratory diseases has been presented. Essentially it is a combination of the chorio-allantoic membrane inoculation technique with the allantoic sac inoculation method. In a comparative study it has been proven a better method for primary isolation of infectious bronchitis virus from field material than the presently accepted allantoic technique.

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PRELIMINARY INVESTIGATIONS ON THE HEMATOLOGY OF BROILER FLOCKS* **

By T. N. FREDRICKSON, H. L. CHUTE and D. C. O'MEARA

Department of Animal Pathology, Maine Agricultural Experiment Station, University of Maine, Oronto, Maine

RELATIVELY little use has been made of clinical hematology by poultry pathologists despite the fact that it is used extensively in the diagnosis and prognosis of many disease conditions in human and veterinary medicine. This apaprent neglect of a potentially useful laboratory aid may be due to two major reasons. Firstly, many difficulties accompany attempts to perform tests on chicken blood which are comparatively simple in mammalian hematology. The second reason is the lack of standard normal ranges for any component of chicken blood because of the paucity and conflicting contents of the reports on avian hematology. Among the reports available are those by Dukes and Schwarte[9], Elvehjem and Schultze[20], Bankowski[1], Cook and Harmon[7,11] and Tanaka and Roseburg[20], dealing with hemoglobin determinations. Blaine[3], Forkner[10] and Palmer and Biely[18] have reported on the cytology of chicken blood. Clinical methods have been reviewed by Olson[17], and Dennington and Lewis[8]. Reports on clinical applications of avian hematology include those by Cook and Dearstyne[5], Blalock[4], Cook[6], Hogan and Parrot[12], Mushett and Washko[13], and Natt and Herrick[16].

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There were two main objectives for this study of chicken blood. The first was to find which hematological tests could best be adapted for routine use on chicken bood. The second objective was to determine how these tests could be applied to practical disease control. When techniques were sufficiently standardized they were applied to determine normal values of broiler-type birds for the following determinations: erythrocyte, leukocyte and thrombocyte counts, hemoglobin, whole blood and plasma specific gravities, erythrocyte fragility, hematocrit, (by the Wintrobe and micro-capillary methods), erythrocyte sedimentation rate and differential leukocyte counts. Bone marrow studies were also done for better understanding of the immature cells found in peripheral blood smears.

METHODS AND MATERIALS

The above tests were conducted on 243 White Rock chickens from one to ten weeks of age. This report, however, deals mainly with 93 samples recently studied by the senior author. After a survey of available methods the following were chosen as the most suitable. The red blood cells were counted in an improved Neubauer hemocytometer using Natt's fluid[15] as a diluent. The white blood cells were diluted with a modified Wiseman's fluid[22,14] which stains the heterophils and eosinophils and lyses all other cells. All the stained cells in 3 cubic mm were counted in a Fusch Rosenthal hemocytometer and the total white blood cell, (WBC), count was calculated by multiplying the number of such cells in 1 cubic mm by the ratio of heterophils and eosinophils to other types of leukocytes as obtained from the WBC differential count. The differentials were done from whole blood smears made on cover slips[21], stained with Wright's and then Giemsa's stain. Despite the difficulties entailed in the cover slip method, it is preferable to the usual one of spreading a drop of blood on a glass slide. Clumping of the monocytes and granulocytes often occurs along the borders of a smear made on a slide. This clumping distorts the differential count. It was found necessary to use both Wright's and Geimsa's stains because while the latter gives a generally satisfactory result, it leaves the

heterophilic rods unstained. Wright's stain gives the classical picture of heterophilic rods when the buffer pH is at 6.0 and the stain-buffer combination is left on the smear for 5 minutes, but it is unsatisfactory for staining granulocytic nuclei. At least 200 leukocytes were enumerated per smear. In the smears in which the thrombocytes were not agglutinated, these cells were counted and their number per cubic mm was expressed as a percentage of the total WBC count. The hemoglobin was determined by the acid-alkali method[1] comparing the acid hematin with a standard using a Klett Summerson photoelectric colorimeter. The hematin solutions were centrifuged at 2,500 for 10 minutes to eliminate erythrocyte nuclei from the hematin solution. Specific gravities for whole blood and plasma were measured in copper sulfate solutions according to the method of van Slyke[21]. The erythrocyte fragility tests were carried out by placing a drop of whole blood in test-tubes containing varying concentrations of saline (NaCl), and noting the range between minimal and maximal resistance to hemolysis. The reading for the hematocrit as given by the Wintrobe and micro-capillary methods were compared. The sedimentation rate was gauged by allowing the Wintrobe hematocrit tube to stand vertically for 1 hour and measuring the mm of plasma cleared of erythrocytes.

RESULTS

Tables I and II show the data obtained in this preliminary study. The work revealed some note-worthy characteristics of blood from normal chickens.

1. It contains many immature cells; lymphoblasts, myelocytes and normoblasts are not uncommon in the blood of even mature birds. Up to two weeks of age chicks have a great number of circulating immature cells, e.g. one to three promyelocytes are usually seen per 200 leukocytes in young chicks. Unless their number is comparatively great, circulating immature cells cannot be considered to have any pathological significance in young chickens. Mitotic normoblasts and lymphoblasts are sometimes seen in the blood of normal chickens, even as old as 10 weeks.

TABLE I

The weighted means found for some components of the blood of White Rock chickens one to ten weeks of age

Age in weeks	WBC /mm³	RBC millions /mm³	Thrombo-cytes /mm³	Hb gm/100ml	Hematocrit cc/100ml micro	Hematocrit cc/100ml Winthrobe	Blood SG†	Plasma SG†	E.S.R.* mm	E. Fragility** min. % NaCl	E. Fragility** max. % NaCl
1	12,500	2.70	25,800	7.1	22.5	23.3	1.038	1.019	4.1	.44	.33
2	12,800	2.64	none recorded	7.5	23.6	28.8	1.042	1.018	2.2	.41	.33
3	16,300	2.22	32,500	8.8	24.5	27.3	1.042	1.019	1.8	.45	.38
4	22,000	2.22	28,000	7.4	23.8	24.9	1.044	1.022	2.0	.42	.33
5	20,000	2.35	28,100	7.8	25.8	27.3	1.042	1.018	1.5	.43	.34
6	17,900	2.01	29,500	7.0	23.1	24.0	1.041	1.018	1.7	.39	.31
7	20,000	2.28	27,500	7.7	23.8	25.3	1.041	1.018	1.6	.38	.28
8	26,300	1.98	30,800	7.2	25.5	25.8	1.044	1.020	1.4	.42	.34
9	23,600	2.19	37,500	7.4	24.6	24.3	1.040	1.020	1.8	.39	.31
10	33,900	2.19	43,800	8.1	24.3	23.4	1.040	1.019	2.0	.41	.31
Mean	20,500	2.08	31,500	7.6	24.2	25.4	1.041	1.019	2.0	.41	.33
No. of samples	78	87	51	92	86	93	93	93	89	93	93

* Erythrocyte sedimentation rate
** Erythrocyte fragility, points of minimal and maximal resistance to hemolysis
† SG = specific gravity

2. Erythrocytes of normal chickens exhibit considerable anisocytosis and also variation in nuclear size, shape and position. Non-nucleated erythrocytes are fairly often seen in normal blood samples.

3. There exists a great degree of individual variation in the blood pictures of normal birds. This conclusion was also reached by Biely and Palmer[2]. It leads us to believe there is no chance to establish narrow ranges for blood component in chickens, but rather that the norms will have to be quite wide ranges. If hematology is to be applied from a practical viewpoint, a large number of samples should be taken because no reliance can be placed on the findings from a few samples.

4. Within the first 10 week period of a chicken's life its blood picture varies considerably according to its age. This is especially true of the WBC count which is much lower at hatching than at 10 weeks of age.

5. The blood of chickens has a pronounced tendency to form micro-clots, i.e. clumping of the thrombocytes. Since these cells are supposedly physiologically similar to platelets in mammalian blood it is not surprising to find that they agglutinate so quickly. While the blood still remains fluid these thrombocytic clumps interfere with some tests and especially the WBC count. When there is moderately severe clumping, the WBC counts are worthless because many leukocytes are enmeshed in the masses of thrombocytes. The only method found to overcome this difficulty was to take blood from a wing vein using a very sharp 2 inch, 20 gauge, hypodermic needle to make the venipuncture. In young chicks diluting pipettes must be filled directly from a punctured vein. The preferable anticoagulant was undiluted heparin*, 0.2 ml of which was sufficient to prevent micro-clots in a 5 ml sample if the barrel of the syringe was thoroughly wetted with heparin and the blood and heparin were mixed immediately.

DISCUSSION

This preliminary investigation indicates to us that there is little chance of applying clinical hematology as it is used in human or mammalian veterinary medicine to avian disease control. A new approach must be made. This is because avian

* Lederle heparin—1 ml contains 1000 U.S.P. units.

TABLE II

WBC differential values in percent of total count and actual number of each type of leukocyte/mm³ for 93 samples from White Rock chickens

Age in Weeks	Small Lympho-cytes	Large Lympho-cytes	Mono-cytes	Myelo-cytes	Eosino-phils	Baso-phils	Hetero-phils
1	20*	55	1	0	0	0	24
	2,500**	6,875	125	—	—	—	3,000
2	22	56	2	3	1	1	15
	2,816	7,168	256	384	128	128	1,920
3	39	29	2	2	1	1	26
	6,357	4,727	326	326	163	163	4,238
4	44	29	3	1	0	1	22
	8,800	6,380	660	220	—	220	4,840
5	40	32	4	1	1	1	21
	10,000	6,400	800	260	200	200	4,200
6	49	20	3	1	0	1	26
	8,771	3,580	537	179	—	179	4,654
7	47	21	4	1	1	5	21
	5,400	4,200	800	200	200	1,000	4,200
8	50	21	4	1	0	2	22
	13,150	5,523	1,052	263	—	526	5,786
9	50	21	3	0	0	1	25
	11,650	4,956	708	—	—	236	5,900
10	43	18	4	1	0	1	33
	14,577	6,102	1,356	339	—	339	11,187
Mean	40.4%	30.2%	3.0%	1.1%	.4%	1.4%	23.5

* Percentage
** Actual Number /mm³

thromocytes clump very readily thus deranging the WBC count. In addition, the turbidity caused by the nuclei of avian erythrocytes precludes a quick, accurate measurement of hemoglobin by photoelectric methods. This is significant because the WBC count and hemoglobin determination are two very important findings in mammalian clinical hematology. Another point to consider is that extreme variation in the blood picture of birds is often associated with changes in environment[18]. This means that birds brought into a diagnostic laboratory will not reflect truly the blood picture of the flock as a whole. Therefore, blood samples must be taken in the field to be really reliable.

The whole blood specific gravity test and the micro-capillary hematocrit are the two tests which could be applied in the field in large enough numbers to overcome the difficulty of wide individual variations in the blood picture. The whole blood specific gravity will give an estimation of the hemoglobin and RBC count, both of which are lowered in many diseases.[4,5,6,12,16,17] The hematocrit gives the percentages of packed erythrocytes in the blood and of the buffy coat. The latter gives a rough idea of the WBC count since the buffy coat includes all the leukocytes and thrombocytes. These two tests, applied in the field, may prove useful as indicators of the health of flocks and as guides to diagnosis and prognosis.

The other tests which were applied in this preliminary investigation did not seem satisfactory from a practical standpoint. The erythrocyte fragility test was difficult to interpret. The Wintrobe method for hematocrit determination necessitated too much equipment for field work, and did not seem to be any more reliable than the micro-capillary method. The erythrocytes in chicken blood settled comparatively slowly and too long a time would have to be spent observing sedimentation rates for this test to be practical.

The fowl is an important experimental animal. When blood is being taken for experimental work it is necessary to take great care in obtaining samples and these samples must be examined immediately. A thorough knowledge of normal chicken's blood picture at any particular age is necessary

before conclusions can be drawn from the study of the blood from diseased birds.

SUMMARY

The blood picture of 93 normal broiler birds from 1 to 10 weeks of age is discussed and methods and its possible application for several hematological tests are described. The value of hematology in avian disease control is discussed.

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A SIMPLIFIED METHOD FOR THE TITRATION OF ANTIGEN IN THE BETA HEMAGGLUTINATION-INHIBITION TEST

By R. F. GENTRY

Department of Veterinary Science, Pennsylvania State University, University Park, Pennsylvania

THE beta or Lederle technique[1] for the hemagglutination inhibition test is employed in many diagnostic and research laboratories. This technique requires less antigen per test than the alpha or BAI method[2] and eliminates the necessity of calculations for the determination of titers of positive serums. However, the accuracy of the beta technique depends entirely upon the concentration of the virus in the antigen. The antigen is titrated using two series of two-fold dilutions, one starting at a 1/20 and the other at a 1/30 dilution as shown in table I. In this titration the highest dilution showing hemagglutination is 1/480. The value of one unit is calculated by averaging the highest dilution in which hemagglutination occurs with the next highest dilution of the other series ($480 + 640 \div 2 = 560$). Therefore a dilution of 1/560 contains one hemagglutinating unit and dilution of 1/70 would contain eight hemagglutinating units in each 0.5 ml. The eight unit antigen is then used to prepare dilutions of the serum for the hemagglutination-inhibition test. If inhibition of hemagglutination occurs at a 1/16 dilution[1], the serum is

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TABLE I

Antigen titration for the beta and Lederle technique

	Tube no.									
	1	2	3	4	5	6	7	8	9	10
Row 1	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
	+	+	+	+	+	—	—	—	—	—
Row 2	1:30	1:60	1:120	1:240	1:480	1:960	1:1920	1:3840	1:7680	1:15360
	+	+	+	+	+	—	—	—	—	—

* + = hemagglutination

considered positive. In some instances, complete inhibition at a serum dilution of 1/8 is considered significant[3]. However, the level of significance is based on the assumption that the antigen contains exactly eight units.

The procedure described in the Lederle circular[1] for determining the eight unit concentration had been used routinely in our laboratory. However, when the antigen concentration determined by this method was checked in the antigen control reaction, the one unit mixture always failed to produce hemagglutination. This antigen control reaction consisted of a series of two-fold dilutions of the antigen prepared so that tubes containing four, two, one, one-half and one-quarter units were present. If the original preparation contained exactly eight units, hemagglutination would occur in the four, two, and one unit tubes but not in the one-half and one-quarter unit tubes. Such a reaction would indicate that there were at least eight units present in the calculated antigen concentration but it could contain much more without the one-half unit tube showing hemagglutination.

If the calculated antigen concentration is too low, hemagglutination does not occur in the one unit tube. In this case the concentration can be adjusted and the material retested until the proper concentration is obtained. However, if the calculated concentration is such that there are more than eight units present, the exact titer is not known since excess antigen can be present and still give a positive reaction in the one unit tube and be negative in the one-half unit tube.

The techique outlined in this report was designed to give a direct determination of the exact concentration containing eight units. This would eliminate the necessity of adjusting the concentration when no reaction was found in the one unit tube of the antigen control test and prevent the possible presence of excessive antigen, which would give inaccurate results in subsequent hemagglutination-inhibition tests.

PROCEDURE AND RESULTS

Salk agglutination plates of the type shown in figure 1 were used for all tests. The individual depressions or re-

ceptacles will be referred to as spots. These plates have the advantage of being easily read from the top and a large number of tests can be read at the same time. Readings are made after 30 minutes incubation at room temperature, or when the red cell control spots (row H, columns 8, 9 and 10) have completely settled. The plate is then tilted at approximately a 30 degree angle and the presence or absence of flowing buttons determined.

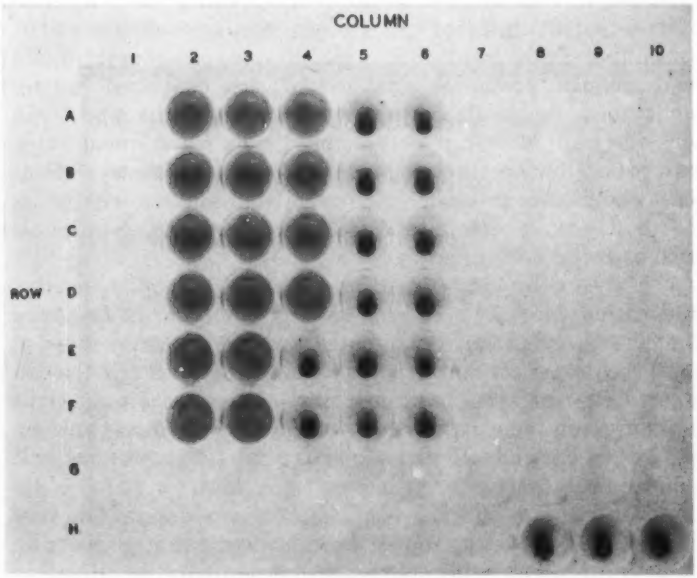

Fig. 1. Antigen titration using a Salk agglutination plate.

Commercial Newcastle disease antigen (diagnostic) was used. A 1/50 dilution was prepared by adding 0.2 ml of the stock antigen to 9.8 ml of phosphate buffer[4], and 1 ml placed in each of the spots A through F of column 1. Varying amounts of buffer were then added as shown in table II giving dilutions of 1/50 through 1/100. If the titer is 1/100 or

greater the appropriate dilutions are used in column 1. A quantity of 0.5 ml of buffer was then placed in the spots of columns 2 through 6 for each of these six rows. Starting with the 1/50 dilution, 0.5 ml was transfered to the next spot across the row. A clean pipette was used to mix the material and transfer 0.5 ml to the next spot. This was continued across each row and the 0.5 ml from the last spot (column 6) discarded. To each of these spots 0.5 ml of a two percent suspension of chicken red blood cells was then added. The red cell control spots (Row H, columns 8, 9, and 10) contained 0.5 ml of buffer and 0.5 ml of the cell suspension. When all materials had been added the plate was rotated carefully to thoroughly mix the contents of each spot, and incubated for approximately 30 minutes at room temperature. When the cell control spots showed compact buttons the test was read.

The purpose of the test is to determine the concentration of antigen containing eight units per 0.5 ml. One of the dilutions prepared in column 1 should thus contain eight units per 0.5 ml. Since these have been carried through a series of two-fold dilutions the spots in column 2 would represent four units, column 3—two units, column 4—one unit, column 5—one-half unit and column 6—one-quarter unit. This of course would only be accurate for the dilution that actually contains eight units, which would be the highest dilutions giving hemagglutination in the one unit column. The results of the test shown in figure 1 are tabulated in table III. The highest dilution giving a positive reaction (hemagglutination) at the one unit level (column 4) is 1/80. Therefore this dilution would contain exactly eight units per 0.5 ml and would be used for subsequent hemagglutination-inhibition tests.

It should be noted that the 1/50, 1/60 and 1/70 dilutions also give a positive reaction in the one unit spot and negative in the one-half unit spot. If any of these dilutions had been tested separately the excess antigen would not have been detected and if used for hemagglutination-inhibition tests, more than eight units of antigen would have been present.

TABLE II

Preparation of dilutions for antigen titration

Row	1:50 Antigen (ml.)	PO_4 Buffer (ml.)	Antigen Dilution
A	1.0	0.0	1:50
B	1.0	0.2	1:60
C	1.0	0.4	1:70
D	1.0	0.6	1:80
E	1.0	0.8	1:90
F	1.0	1.0	1:100

TABLE III

Tabulation of antigen dilutions used in the simplified technique and results of titration in figure 1.

Row	* 1	2	3	4	5	6	† 7	† 8	† 9	† 10
A	O 1:50	+ 1:100	+ 1:200	+ 1:400	— 1:800	— 1:1600				
B	O 1:60	+ 1:120	+ 1:240	+ 1:480	— 1:960	— 1:1920				
C	O 1:70	+ 1:140	+ 1:280	+ 1:560	— 1:1020	— 1:2040				
D	O 1:80	+ 1:160	+ 1:320	+ 1:640	— 1:1280	— 1:2560				
E	O 1:90	+ 1:180	+ 1:360	— 1:720	— 1:1440	— 1:2880				
F	O 1:100	+ 1:200	+ 1:400	— 1:800	— 1:1600	— 1:3200				
G								**		
H								—	—	—
Unit Value	8	4	2	1	½	¼				

* Column 1 used for preparation of antigen dilutions. No red cells added.

** Row H column 8, 9, 10, red cell control.

† Column 7, 8, 9, 10 not used for titration.

A comparison of the Lederle method and the one described here may be more easily understood if the unit values are interpreted as antigen dilutions. In table III the actual antigen dilutions are also given. If the test were read and the one unit spot determined, it would be row D of column 4 or 1/640 and the eight unit dilution would be 1/80. It is readily

seen that the interval between dilutions is much less in this method than in the Lederle method shown in table 1. The end point therefore would be more accurate. Also, the highest dilution in a column (row F) is the same as the lowest dilution in the next column (row A). These spots must always give the same reading thus serving as a check on the procedure.

SUMMARY

An accurate and direct method for determining the eight unit antigen level for the hemagglutination-inhibition test is described.

1. Only 0.2 ml of stock antigen is required for the titration compared to at least 1 ml required by the Lederle method leaving more antigen available for hemagglutination-inhibition tests.
2. The exact titer is determined directly, making it unnecessary to check and adjust the concentration.
3. The use of more dilutions at smaller intervals eliminates the possibility of excess antigen being present in subsequent hemagglutination-inhibition tests.

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PRELIMINARY BLOOD STUDIES IN CHICKENS WITH A SYNOVITIS CAUSED BY THE INFECTIOUS SYNOVITIS AGENT, PLEUROPNEUMONIA-LIKE ORGANISMS AND A COMBINATION OF THE TWO AGENTS [1] *

By N. O. OLSON, D. C. SHELTON, D. A. MUNRO
and RUTH BLETNER
West Virginia University, Morgantown, West Virginia

THE blood changes in birds with infectious synovitis have been described[4,5]. Generally, birds with infectious synovitis had less hemoglobin, lower erythrocyte counts and higher leucocyte counts than normal. The differential leucocyte counts revealed a decrease in the percentage of lymphocytes, increase in heterophils, monocytes and "preforms". The latter were promonocytes, prolymphocytes, myelocytes or the blast forms of these cells. The red cells were achromic and had polychromatophilia, anisocytosis or poikilocytosis. Occasional, and sometimes numerous, immature erythrocytes were present.

This study was undertaken to compare blood changes in chickens with a synovitis caused by the infectious synovitis agent, and a synovitis caused by pleuropneumonia-like organ-

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isms (PPLO) or a combination of these agents. If marked differences occur in the blood changes caused by these two diseases, such differences could be used in making a differential diagnosis.

METHODS

The New Hampshire chicks used in these studies were reared in isolation cages. The chicks were fed the ANRC standard control chick and broiler diet[2]. Before inoculation the serum from each of 12 chicks checked for chronic respiratory disease antibodies with a plate PPLO antigen was negative.

The agents used in these trials were the infectious synovitis agent 29M[4], and the turkey sinusitis agent 1730-3[3] isolated in this laboratory. For inoculation of the chickens, yolk cultures of the second embryo passage were used. Both agents had been passed once through chickens. The embryo cultures of the turkey sinusitis agent will be referred to as PPLO embryo yolk cultures and broth cultures will be referred to as PPLO broth cultures. No PPLO were isolated from the infectious synovitis embryo cultures.

PPLO were isolated in broth from the purulent synovial exudate of chickens inoculated with the PPLO embryo yolk cultures. The second and the fourth passages of this broth culture were inoculated into chickens to check the results obtained from PPLO embryo yolk cultures.

The chicks infected with yolk cultures were bled by venipuncture, two to three times weekly for a period of 37 to 38 days. When PPLO broth cultures were used, the hock inoculated birds were bled 10, 18, and 21 days after inoculation. The pad inoculated birds were bled 1, 3, 7, 9, 13, 17, and 29 days after inoculation. Hemoglobin was determined by the acid hematin method. Erythrocytes and leucocytes were counted using the method of Natt and Herrick[1]. The differential leucocyte counts were made from May-Gruenwald and Giemsa stained blood smears. Birds were weighed and observed each time they were bled.

RESULTS

The number of birds inoculated, number infected and

the number of deaths are shown in table I. The influence of the infective agents on the blood components of birds exposed by foot-pad inoculation to embryo yolk cultures are shown in figures 1 to 7. In these figures the values for the contact controls are averages obtained from those contact controls which did not develop synovitis. Only three contact controls became infected and these were in contact with other birds which were infected with the infectious synovitis agent by sinus inoculation. The blood changes observed in the birds infected through contact or sinus inoculation are not shown since they were similar to the changes in pad inoculated birds.

When the PPLO embryo yolk cultures were used, none of the contact controls or the sinus inoculated birds developed a synovitis. Only pad inoculated birds showed signs of synovitis. The same was true when PPLO broth cultures were used. The results from birds inoculated with PPLO broth cultures are not shown. However, the following values were observed to be the most severe changes for these birds: hemoglobin, 7.6; erythrocyte count, 2,400,000 per cubic mm.; leucocyte count,

TABLE I

Foot-pad and sinus inoculation of birds with infectious synovitis and PPLO

INOCULUM	Route	Age weeks	Number of birds	Number with synovitis	Number died
Infectious synovitis	Foot-pad	3	4	4	3
None—contact controls	——	3	4	0	0
Infectious synovitis	Sinus	3	4	2	1
None—contact controls	——	3	6	2	0
PPLO yolk culture	Foot-pad	3	4	4	0
None—contact controls	——	3	4	0	0
PPLO yolk culture	Sinus	3	4	0	0
None—contact controls	——	3	5	0	0
PPLO* + Infectious synovitis	Foot-pad	3	4	4	3
None—contact controls	——	3	4	0	0
PPLO* + Infectious synovitis	Sinus	3	4	3	2
None—contact controls	——	3	4	1	1
PPLO 6-day broth culture (4P)	Foot-pad	7	8	8	0
PPLO 9-day broth culture (2P)	Hock	3	6	6	0

* yolk culture

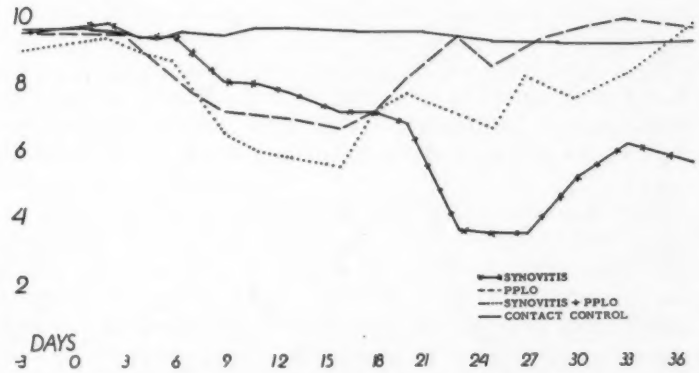

Fig. 1. Hemoglobin in grams per 100 ml blood of chickens infected with synovitis, PPLO and a mixture of both.

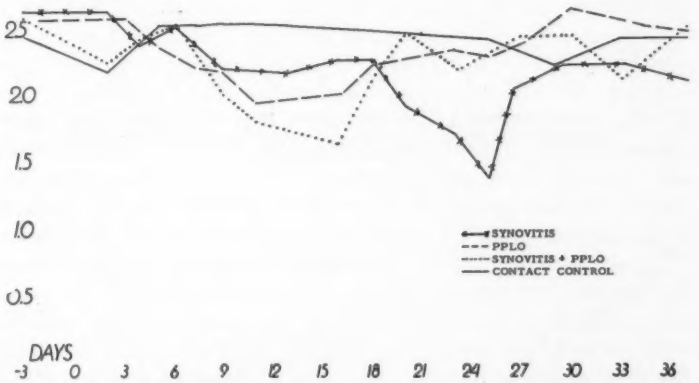

Fig. 2. Erythrocyte count in millions per cubic ml of blood from chickens infected with synovitis, PPLO and a mixture of both.

86,000 per cubic mm.; the differential count percentages were lymphocyte, 29; heterophil, 57; monocyte, 15; "preforms" 4.5.

In the pad inoculated birds, one bird died the 12th, 18th, and 22nd day after inoculation with infectious synovitis and on the 13th, 16th and 18th day after inoculation with infectious synovitis plus PPLO embryo yolk cultures. No deaths

Fig. 3. Leucocyte count in thousands per cubic ml of blood from chickens infected with synovitis, PPLO and a mixture of both.

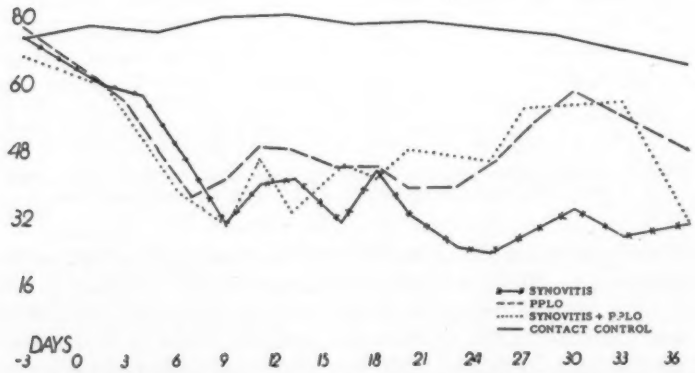

Fig. 4. Lymphocyte percentage of chickens infected with synovitis, PPLO and a mixture of both.

occurred in the birds inoculated with only the PPLO embryo yolk cultures. One bird died 23 days after sinus inoculation with the infectious synovitis agent and two birds died 23 days after sinus inoculation with infectious synovitis plus PPLO embryo yolk cultures. One contact control died 33 days after exposure of the pen mates to infectious synovitis plus PPLO embryo yolk cultures.

The reduction in hemglobin and erythrocytes was associated with the severity of the synovitis. Generally the hemoglobin and erythrocyte counts were lower with infectious synovitis than with PPLO synovitis. The combination of infectious synovitis and PPLO synovitis lowered hemoglobin and erythrocytes counts more than either of these diseases alone. Also these reductions were lower with yolk cultures of PPLO than with broth cultures of PPLO. There was no marked reduction in erythrocyte counts in the initial trials when PPLO broth cultures were used. These differences might be explained in part by the severity of the infection.

The morphology of the red cells varied with the severity of the infection as did the decrease in hemoglobin and erythrocyte count. The erythrocytes in severely affected birds showed anisocytosis, poikilocytosis, polychromatophilia and achromia. Immature erythrocytes of varying degrees were present. The anemia present in these birds was a secondary anemia associated with either infectious synovitis, PPLO synovitis, or a combination of these two diseases.

The synovitis produced in sinus inoculated birds and in contact controls was due to the infectious synovitis agent. The changes in the blood picture in these birds was associated with the development of infectious synovitis which occurred at about the 14th day in sinus-inoculated birds and about the 24th day in contact controls. The birds inoculated by the way of the sinus with the embryo cultures of PPLO did not develop a synovitis. These birds developed a sinusitis in 11 to 18 days and the contact controls in these pens developed a sinusitis in 24 to 35 days. In previous experiments using PPLO embryo cultures, a synovitis developed in a few birds following sinus inoculation[3].

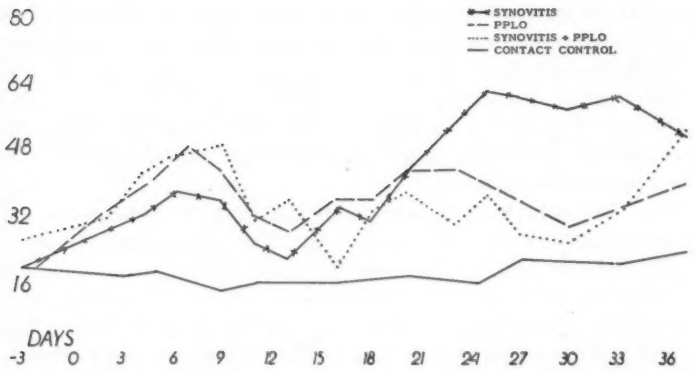

Fig. 5. Heterophil percentage of chickens infected with synovitis, PPLO and a mixture of both.

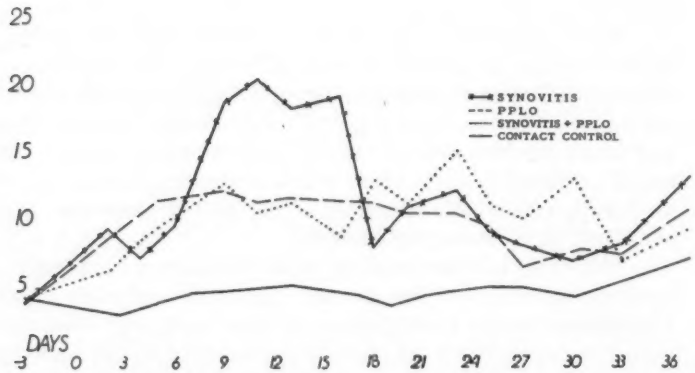

Fig. 6. Monocyte percentage of chickens infected with synovitis, PPLO and a mixture of both.

The leucocyte counts from foot-pad inoculated birds began to increase 2 to 4 days after inoculation and became higher as signs of synovitis increased. One to two days before death, the leucocyte count reached its maximum. When partial recovery was apparent, the counts decreased. With all

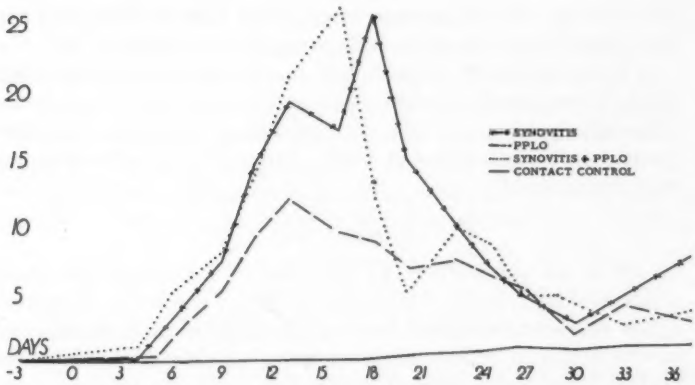

Fig. 7. White blood cell preform percentage of chickens infected with synovitis, PPLO and a mixture of both.

routes of inoculation and in infected contact controls, an increase in the leucocyte count occurred 2 to 4 days before signs of synovitis appeared. There were no marked differences in the total leucocyte count between birds treated with the various inocula, although in a few cases the counts were higher in birds inoculated with the synovitis agent.

When the total leucocyte count increased, the percentage of lymphocytes decreased and the percentage of monocytes and heterophils increased. This shift in the differential count appeared shortly before signs of synovitis developed. The "preforms" of leucocytes in pad inoculated birds started to increase on the 6th to 9th day and generally reached a maximum on the 14th to 18th day. The changes were similar with each agent, although the greatest percentage of "preforms" occurred in birds inoculated with the infectious synovitis agent and the infectious synovitis agent plus PPLO embryo yolk cultures. The percentage of monocytes was highest in the birds with infectious synovitis.

The "preforms" were not defined in the differential counts. However, attempts were made to identify them in a few of the severely affected birds. The cell forms found were

classified as promonocytes, myelocytes and prolymphocytes. The blast forms of these cells were also present.

Toxic granules were noted in the heterophils in some birds. These appeared more frequently in the birds inoculated with PPLO. Also, granuloctic myelocytes appeared more frequently in PPLO infected birds. Their significance has not been determined.

DISCUSSION

It is not the purpose of this paper to compare the signs of infectious synovitis with those of PPLO synovitis; however, a few differences should be mentioned. The caseous exudate in the synovial membranes, extreme emaciation and dehydration, frequently seen in infectious synovitis has not been found in PPLO synovitis. Breast blisters occurred in nearly all cases of infectious synovitis, but were not common in PPLO synovitis. Splenomegaly, enlarged kidneys and mottled or discolored livers have not been found in PPLO synovitis. Birds inoculated into the sinus with PPLO cultures generally develop a sinusitis and rarely a synovitis. A synovitis in contact controls has not occurred in birds exposed by contact with birds inoculated with PPLO cultures.

Synovitis in birds caused by the agent was generally more severe than that caused by PPLO. The reduction in hemoglobin and erythrocytes was greater in birds with infectious synovitis than in birds with a PPLO synovitis. The differential count was similar, but there were more monocytes and "preforms" in blood smears from birds with infectious synovitis than in blood smears from birds with PPLO synovitis. The differences that occurred might be explained in part by the severity of the infection. To accurately assess the importance of these differences, further experimentation involving a larger number of birds is needed.

Leucocyte counts of 30,000 to 60,000 were found in some of the contact controls that had no signs of infectious synovitis. A satisfactory explanation for these higher counts cannot be made at this time. Considering the leucocyte counts shown in figure 3, the increase was gradual during the experimental period. The differential counts in these birds was within normal range.

SUMMARY AND CONCLUSIONS

The hemoglobin values and erythrocyte, leucocyte and differential leucocyte counts are given for birds infected with infectious synovitis, PPLO synovitis and a combination of these two diseases. These two diseases could not be differentiated on the basis of blood counts or hemoglobin values. In this experiment, hematological changes associated with either disease varied with the severity of the condition.

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A DOUBLE SERUM NEUTRALIZATION TEST TO INDICATE THE RELATIVE LIVE VIRUS CONTENT OF INFECTIOUS BRONCHITIS VACCINE. *

By D. C. O'MEARA

Department of Animal Pathology, University of Maine, Orono, Maine

THE pressing need for a quick test to indicate the relative live-virus content of infectious bronchitis virus (IBV) vaccine prompted the present study. The test outlined is a modification of the SN (serum neutralization), serum dilution test described by Crawley[1] to indicate a change in the titer of a known antiserum after reaction with a vaccine sample.

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For the formulation of this test, the following assumptions were made: (1) the antibody response measured by the SN test is the same antibody which responds to field strain IBV vaccine, and is produced as a result of exposure to this virus; (2) the antibody-field strain virus neutralization will prevent the neutralization of the egg adapted Beaudette the relative amount of virus present in the IBV field strain vaccine sample.

METHODS

Pooled IBV antisera were diluted tenfold aseptically in 500 ml amounts of sterile buffered saline pH 6.8 containing 50,000 units of penicillin G potassium and 0.2 grams of streptomycin sulfate. The vessel containing the dilution was then inverted 10 times before a portion was removed for the next dilution. These dilutions were then dispensed in 20 ml amounts to facilitate use. Storage was at 20 C.

A preliminary titration was made on the serially diluted serum with the egg adapted IBV. Serum and virus (AA fluid) were added in a one to one volume ratio to sterile tubes, shaken and inverted to mix and allowed to react for 20 minutes at 4 C. Ten-day embryonated chicken eggs were injected in the allantoic cavity with 0.2 ml of the virus antiserum mixture. Five eggs were used for dilution. The eggs were candled at 24 and 48 hours and the LD_{50} was observed based on mortality at 48 hours not due to bacterial contamination.

The vaccine sample was reconstituted according to the manufacturer's direction and 0.2 ml injected into six ten-day-old embryonated eggs. This passage was made to propagate the live virus present in the vaccine. The AA fluid harvested after 48 hours incubation was tested for sterility and pooled. This pooled sample was added to at least three antisera ten fold dilutions on either side of the LD_{50} dilution found above at a volume ratio of one-half sample to one of antisera. The mixtures were mixed by inverting the tubes and allowed to remain for twenty minutes at 4 C to react. The egg adapted

IBV was then added to each dilution used in a one-half volume ratio and mixed. A second neutralization of twenty minutes at 4 C was made.

A control using the same six dilutions of antiserum and egg adapted virus in a one to one volume ratio was made with each run of samples. This mixture was subjected to the second neutralization of twenty minutes at 4 C. Following neutralization, 0.2 ml from each control and from each sample dilution was injected into at least three ten-day-embryonated eggs via the chorio-allantoic route. Injected eggs were incubated at 38 C, and candled at twenty-four and forty-eight hours. The deaths at 24 hours were considered due to trauma. An estimation of the LD_{50} was made on the mortality at 48 hours by the method of Reed and Meunch.[2]

When there was no difference in the embryo mortality pattern at forty-eight hours between the control set of dilutions and the sample set, the vaccine virus was considered to have been of the same titer as the egg adapted virus used. Likewise, a shift of the LD_{50} up or down the antiserum scale would indicate a corresponding shift in the titer of the virus being tested.

The results obtained to date, using six vaccine samples (157 dozen eggs) have not been consistent. Fluctuations seem to indicate an auto-interference mechanism such as Groupe[3] encountered. This may be due either to a disassociation of sample virus-antibody combination or to a utilization of more receptor groups of the antibody under certain conditions. The single embryo passage appeared necessary to select the live virus. However, error may be introduced by the inability of the virus to establish itself or because of unusual multiplication and consequent erroneous titer to occur.

The widespread switch to a combination vaccine by poultry producers added another difficulty. A preliminary absorption with anti-Newcastle serum is necessary before the single egg passage of the vaccine under investigation is done. The need for some means more specific than dwarfing or curling of embryos or the vaccination of immune birds and the subsequent wait for an SN titer to indicate the IB virus con-

tent of vaccine exists. The test described may, with further refinement, indicate the relative live virus content of IB vaccine.

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LABORATORY ANALYSIS OF SEVERAL FIELD OUTBREAKS OF CHRONIC RESPIRATORY DISEASE *

By J. F. SULLIVAN, E. GILL, A. SOMER, and K. HEDDLESTON

Animal Disease and Parasite Research Divison, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

NUMEROUS reports have been published regarding the incidence and occurrence of disease-producing agents other than pleuropneumonia-like organisms (PPLO) in field outbreaks of chronic respiratory disease (CRD).[1,2,4,6] It is the purpose of this paper to add to the available information pertaining to this subject and to present an account of our

* Presented at the 28th Annual Meeting of the Northeastern Conference of Laboratory Workers in Pullorum Disease Control, June 19 and 20, 1956, Newark, Delaware.

experience with the serum plate test employing commercially prepared PPLO antigen.

MATERIALS AND METHODS

Six farms, located in the States of Delaware, Maryland, and Virginia, were visited during the course of this study. To correlate the results of serological tests for PPLO with the time lapse since the onset of symptoms, the birds on three of these farms were subdivided into two flocks. On farms 1 and 4, the flocks were housed in separate units of comparable construction. The birds on farm 5 were housed at opposite ends of a single building, where they were separated by dual wire barriers on each side of a centrally located aisle.

At each visit a number of birds exhibiting symptoms suggestive of CRD were killed and examined for gross pathological lesions. Tissue samples obtained from two to four affected birds were collected individually for laboratory analysis. All such samples were subjected to several laboratory procedures designed to isolate and identify the agents causing the most common respiratory diseases of poultry.

In an effort to demonstrate the presence of PPL organisms, commercially prepared PPLO agar enriched with 2 percent serum fraction, 7-day-old embryonating chicken eggs, 6 to 12-week-old chickens, and 3 to 5-month-old turkey poults were inoculated with suspensions of macerated tissues.

Virus isolations were attempted by means of at least three serial passages in 10 or 11-day-old embryonating chicken eggs. All inoculations were made into the allantoic cavity, according to the method of Cunningham.[3]

Specimens for bacteriological examinations included material taken from the eye, sinus, trachea, nasopharynx, and liver. This material was inoculated into beef infusion broth and agar enriched with 10 percent chicken serum. Cultures obtained from birds with swollen sinuses were injected into the infraorbital sinuses of adult birds having no history of respiratory disease to facilitate the isolation and identification of *Hemophilus gallinarum*. The majority of broth cultures were streaked on McConkey's agar.

Pooled blood samples obtained in the field were tested

for the presence of antibodies against Newcastle disease (ND) and infectious bronchitis (IB) by means of serum-neutralization tests. Serum plate agglutination tests (Lederle antigen, experimental lot) for PPLO were performed on all samples in the manner described by Jungherr *et al.*[5]

RESULTS

Our results with regard to field infections are presented in table I. A summary compiling the results of the examination of individual specimens for PPLO indicating the efficiency of the various procedures is presented in table II.

The discrepancy observed when the flock vaccination history is compared with the results of the serological tests of field samples for infectious bronchitis and Newcastle disease should be noted.

Although all flocks were vaccinated for Newcastle disease, significant quantities of neutralizing antibodies (1,000 neutralizing doses or more) were demonstrated in only six of the nine flocks examined. A B_1-like strain of Newcastle disease virus was recovered from two serologically positive flocks, one of which exhibited nervous symptoms highly suggestive of Newcastle disease infection. Repeated attempts to isolate a virus possessing neurotropic properties from this flock were unsuccessful.

While only two flocks on a single farm were vaccinated against infectious bronchitis, significant serum titers were demonstrated in eight of the nine flocks examined (100 neutralizing doses or more). On the single farm where a bronchitis vaccine was employed, the initial symptoms of the CRD outbreak were at first thought to be post-bronchitis-vaccination reaction.

Colonization of PPLO on a commercially prepared medium (Difco) was accomplished from five of 21 individual tissue samples, representing four of the nine flocks examined.

In the embryo and bird inoculation trials as recorded in table I, it should be pointed out that the results represent recoveries made on a flock basis and, as such, are substantially higher than recoveries made on an individual basis as recorded in table II. In our limited experience with the serum

TABLE I.

TABLE I.

Analysis of several field outbreaks of CRD

Flock History							Laboratory analysis of field samples										
							Serological tests			Isolations							
										Virus	PPLO						Bacteria
														Post-exposure serology chickens			
Farm Number	Flock Number	Age in weeks	Duration of symptoms in days[1]	Percent mortality	Newcastle vaccination	Bronchitis vaccination	Newcastle disease[2]	Infectious bronchitis[3]	PPLO	In embryos	Colonization on agar	In chicken embryos[4]	In turkeys[5]	In chickens[6]	11-13 days	21-23 days	31-33 days	
1	1	14	14	30	+	—	+	+	—	—	+	+	+	+	—	—	....	*E. coli*
1	2	14	2	0	+	—	+	+	—	—	—	+	+	+	—	+	....	*H. gallinarum* *P. gallinarum* *E. coli*
2	3	8	10	10	+	—	+	+	—	ND	—	+	+	+	....	—	+	*E. coli* Pseudomonas
3	4	6	7	3	+	—	—	—	—	—	—	+	+	+	....	+	+	*P. mirabilis* *P. mirabilis* *E. coli*
4	5	9	30	30	+	—	—	+	+	—	—	+	....	+	—	+	+	*Pseudomonas* *P. vulgaris* *E. coli*
4	6	9	10	30	+	—	+	+	—	—	+	+	....	+	—	—	+	*P. vulgaris* *E. coli*
5	7	8	30	20	+	+	+	+	+	—	+	+	....	+	+	+	+	*E. coli* *P. vulgaris*
5	8	8	7	22	+	+	+	+	—	ND	—	+	....	+	—	—	+	*E. coli*
6	9	11	60	27	+	—	—	+	+	—	—	+	....	+	+	+	+	*E. coli*

[1] Time interval between onset of symptoms and farm visit.
[2] Positive serological test for Newcastle, serum titered 10^{-3} or more.
[3] Positive serological test for infectious bronchitis, serum titered 10^{-2} or more.
[4] Embryo lesions or mortality suggestive of PPLO.[5] Symptoms and lesions suggestive of CRD.
[6] Symptoms and lesions suggestive of CRD, confirmed by post-exposure serology.

+ = positive test
— = negative test

TABLE II

Results of examination of individual sepcimens for PPLO

	Isolation of PPLO				Serological tests for PPLO (Plate agglutination test)				
	Laboratory technique		Bird inoculation[1]		Field serum samples		Postexposure serum samples (chicken)[2]		
	On media	In embryos	Turkeys	Chickens	2-14 days[3]	30-60 days	11-13 days	21-25 days	31-33 days
No. of specimens examined	21	21	13 (25)[4]	21 (98)	14	6	15	15	14
No. of specimens positive	5	16	10 (16)	20 (63)	0	5	2	5	14
Percent specimens positive	24	76	77 (64)	95 (64)	0	83	13	33	100

[1] Positive tests were recorded when one or more birds in the group exposed to field material developed symptoms or lesions suggestive of CRD.

[2] All figures represent results obtained when testing groups of 4 or more birds per specimen.

[3] Interval since onset of symptoms when field specimens were obtained: in the laboratory, interval between bird exposure and testing.

[4] Figures in parenthesis represent total number of birds used in each test.

plate agglutination test for PPLO, the results were proportional to the time lapse between the onset of symptoms and the time of testing. In all instances, when an interval of 30 days or more was allowed to elapse between these dates, all tests were positive. At shorter intervals the results were variable, generally being proportional to the interval of time since the onset of symptoms.

Several species of bacteria were isolated. Those identified were *H. gallinarum, Pasteurella gallinarum, Escherichia coli, Proteus vulgaris, Proteus mirabilis,* and an undetermined species of *Pseudomonas.* The specific flocks from which these agents were recovered are indicated in table I.

DISCUSSION

Evidence of the presence of PPLO was found in birds obtained from nine flocks undergoing a sequela characterized by respiratory involvement. In all such outbreaks, evidence of past or concurrent bacterial or viral infections was also found.

A comparison of the vaccination history with the results of virus recovery attempts and serological tests on samples obtained in the field suggests that the present Newcastle disease vaccination procedures do not always produce an adequate duration of immunity. The frequent finding of significant antibody titers against bronchitis virus in flocks having no known exposure to this agent indicates the widespread distribution of this virus in nature. These findings, in flocks undergoing frank outbreaks of a respiratory disease from which PPLO were recovered, may be taken as additional evidence of the ability of viral infection, as well as vaccination procedures, to incite or influence the course of CRD in the field. In a like manner, the results of bacteriological examinations of specimens obtained from such flocks demonstrate anew the frequent simultaneous occurrence of PPLO and bacterial infections. Our experience with the serum plate agglutination test for PPLO, although limited, indicates that the time interval between the onset of symptoms and the time the serum sample is obtained will materially affect the outcome of the test.

SUMMARY

The results of laboratory analysis of birds from nine flocks where outbreaks of CRD existed are presented. In all cases evidence of the presence of multiple etiological agents could be demonstrated. The agents demonstrated include both bacterial and viral agents that have previously been reported as influencing the course of CRD. The results of our limited experience with a commercially prepared PPLO antigen are presented.

ACKNOWLEDGEMENT

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A COMPARISON OF FOUR *IN VITRO* METHODS FOR THE ISOLATION OF THE PLEUROPNEUMONIA-LIKE ORGANISM OF CHRONIC RESPIRATORY DISEASE FROM TRACHEAL EXUDATE * †

By J. R. E. TAYLOR, JULIUS FABRICANT, and P. P. LEVINE

Department of Pathology and Bacteriology
New York State Veterinary College, Ithaca, New York

IN a previous report[8,9] on a study of methods of isolation of the pleuropneumonia-like organism (PPLO) associated with chronic respiratory disease (CRD), it was determined that an *in vitro* procedure held several advantages over other techniques. At that time a comparison was made of a modification of the Grumble's medium[5], the yolk-sac inoculation method[3,4], and solid medium cultivation. The first method not only yielded the largest number of isolations, but controlled bacterial and mycotic contamination most satisfactorily. Other advantages of this medium were speed, simplicity and economy.

The work reported here involved a comparison of the modified Grumble's technique[8,9] with methods outlined by Edward[2], Adler[1] and Lecce[6].

MATERIAL AND METHODS

One hundred thirty-seven tracheas were examined, the

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majority from field cases of respiratory infection of unknown etiology submitted to four of the New York State Poultry Disease Laboratories for diagnosis. Some of the tracheas were from birds on our experimental farm, all of which proved free of PPLO.

Immediately following euthanasia the entire tracheas were removed from the birds and frozen at approximately –20 C until cultured. The period of storage varied from 2 to 53 days.

The exudate and as much of the surface epithelium as possible were removed from each thawed trachea by vigorous rubbing with a sterile cotton swab on a sterile applicator stick. The swab and applicator stick were then introduced into a tube containing approximately five ml of Difco nutrient broth (pH 6.8) and agitated thoroughly to suspend the adhering tracheal contents. The resulting suspension was thoroughly mixed with a one ml pipette which was then used to transfer approximately 0.2 ml to each of the following: (a) a tube of Adler's medium, (b) a tube of Edward's medium, (c) two tubes of modified Grumble's medium (containing dextrose and lactose respectively), (d) a plate of Lecce's medium. The inoculum on the last medium was distributed over the entire surface of the plate with a sterile, bent glass rod.

After 24 hours of incubation at 37 C, 0.2 ml was transferred from Adler's medium to one tube each of dextrose and lactose of the modified Grumble's and to a plate of Lecce's medium. The same procedure was carried out with the inoculum incubated in Edward's medium. This was done in each case to determine if PPLO had propagated in either of these two enrichment media. If fermentation occurred in any of the carbohydrates, the presence of PPLO was confirmed by microscopic examination of Gram-stained smears, and by the inoculation of five of the carbohydrates used in the original study[8,9].

All negative cultures were discarded after 14 days of incubation at which time Gram-stained smears were made. The agar plates were examined at 48 or 72 hours as convenient.

RESULTS

Of the 137 tracheas examined, PPLO were isolated from

50 tracheas by one or more of the four methods studied. Table I is a summary of the number of isolations accomplished by each method. Table II is a comparison of the PPLO isolations obtained from individual tracheas by the four cultural methods.

TABLE I

A summary of the total isolations of PPLO obtained by each method employed.

Culture method	Number of PPLO isolations
Modified Grumble's	49
Adler's	43
Edward's	27
Lecce's	5

TABLE II

A comparison of PPLO isolations obtained from individual tracheas examined by four cultural methods.

Successful culture method	Number of tracheas
A*, E, MG and L	4
A, E, and MG	21
A, and MG	16
A, and E	1
A, MG, and L	1
E, and MG	1
MG alone	6
Total number of tracheas from which isolations were accomplished	50

* A—Adler's medium; E—Edward's medium; MG—Modified Grumble's medium; and L—Lecce's medium.

DISCUSSION

It is to be noted that of the 50 isolations made, the modified Grumble's procedure was successful in 49 cases, failing only on one occasion, in which case an isolation was made by both Adler's and Edward's media. Conversely, the modified Grumble's method was successful on 6 occasions where no other method yielded an isolation, and on 39 occasions where one or more of the other methods failed to produce an isolation.

This work also indicates that the incubation of inocula in Adler's or Edward's media for 24 hours before transfer

into the modified Grumble's medium does not increase the rate of isolations as compared to direct inoculation of the modified Grumble's medium.

The observation has been made that the PPLO of CRD may be an L form of a bacterium[7]. These studies in no way contradict this observation, as each of the media studied contained adequate inhibitors to prevent the development of any bacterial forms. No attempt was made here to shed light on the nature of the CRD agent, but rather to provide a comparison of methods of isolation.

CONCLUSION

It is concluded that the primary isolation of PPLO from tracheal exudates is most successfully accomplished by cultivation in the modified Grumble's medium. It is a simple method even in the hands of workers not familiar with the intracacies of PPLO cultivation.

ACKNOWLEDGMENTS

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BACTERIAL CONTAMINANTS IN POULTRY LIVE VIRUS VACCINES OF EGG EMBRYO ORIGIN *

By J. E. WILLIAMS

Program Services Section
Animal Disease Eradication Division
Agricultural Research Service
Washington, D. C.

LABORATORIES engaged in the production of viable virus vaccines for poultry recognize the importance of constant attention to the problem of bacterial contaminants that may gain access to these products during one or several stages of production. The very nature of the vaccines precludes any treatment that might render the products bacteriologically sterile. Lyophilization further preserves contaminants that may be present; however, it has the advantage of preventing multiplication which occurs in an improperly handled non-lyophilized product.

A large volume of total live virus vaccine production is presently represented by poultry vaccines including those against Newcastle disease, laryngotracheitis, fowl pox, pigeon pox, and infectious bronchitis. Vaccine manufacturers have shown a very active interest in the establishment of better standards through the appointment of a committee by the Veterinary Biological Licensees Association to give attention to this problem. Poultry pathologist groups (Johnson *et al.*[7], Hanson[6], Brandly[1]) are also active in this field and have recommended that bacteriologically sterile products should be

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required. Vaccine manufacturers in general have indicated the difficulties in meeting such a requirement. Furthermore, the necessity of having a bacteriologically sterile product has been questioned. It is pointed out that because of the methods of administration, the vaccines usually become contaminated soon after being put into use.

Regulations relating to the production of viable, virus vaccines for veterinary use do not require that these vaccines be bacteriologically sterile. Moreover, there are at present no minimum, well-defined standards regarding the number of contaminating bacteria that may be present in the produets previous to marketing; however, many maufacturers do make bacterial counts on each lot of vaccine produced and the product is passed or rejected, depending upon results. A bacterial count that may be acceptable to one firm may not be tolerated by another. Thus, present standards are rather arbitrary and variable.

While regulations do not require that the vaccines be bacteriologically sterile, they do require that they be free of organisms considered to be pathogenic. It is well known that pathogenicity is a relative thing and subject to considerable variability. For example, limited numbers of cocci and coliforms may be tolerated in a lot of Newcastle wing-web vaccine. It is known that both of these bacterial types may be egg transmitted from hens with chronically infected ovaries and under such circumstances would be regarded as chicken-adapted types. These organisms when introduced into susceptible fowl might readily set up a focus of infection in almost any organ of the body. It has been shown that while such infections may produce no mortality, they are capable of contributing to low prductivity and complicating other disease problems. Moreover, such organisms share antigens in common with *Salmonella pullorum,* as well as other *Salmonella,* and can cause confusing cross-reactions to serological tests for these diseases (Garrard *et al.*[4,5], Carpenter *et al.*[3], Burton and Garrard[2]). Organisms of the genus *Paracolobactrum* (paracolons) have pathogenic properties in many cases similar to those of the *Salmonella.* These organisms may also be

egg-borne, and their presence in vaccines is definitely undesirable.

Organisms of the *Salmonella* group present the greatest problem of all egg-borne pathogens. Standard procedures (Biological Products Memorandum 54-1, U.S.D.A., A.I.Q. Division, Washington 25, D. C.) are presently available for detecting these organisms during vaccine production. Satisfactory and routinely adaptable procedures are yet to be developed for the detection of other organisms that might be considered pathogenic. One might question the feasibility of identifying all bacterial types that may be encountered on plating out a sample of vaccine.

Neither is the injection of animals with the finished product an absolute safety test for the detection of pathogenic bacteria. The age of the birds and various environmental factors have a very decided influence on their response following exposure to a pathogenic agent. Infections may be incited that are not detected unless the apparently healthy animals are thoroughly examined. Furthermore, infections may not be discernible until they are transmitted through the egg or directly to other susceptible fowl.

Most bacteria present in vaccines of egg-embryo origin are introduced through the use of eggs containing the organisms either internally or on the surface of the egg shell. Internal infection of the egg may result from direct ovarian transfer or from organisms present on the surface of the egg penetrating the shell. The latter route is probably more common.

The fecal origin of contaminating organisms is substantiated by the types of organisms that are encountered in the finished products. During the examination of more than 100 lots of egg-embryo vaccines in our laboratory, approximately 50 percent were found to contain organisms of the genera Proteus, Alcaligenes, Escherichia, Aerobacter, Pseudomonas, Micrococcus, Bacillus, as well as others known to be of fecal origin. Various fungi were also encountered. These findings emphasize the necessity of using for vaccine production only clean, properly handled eggs obtained from pullorum-typhoid

clean flocks and with no history of other *Salmonella* infections. Bacterial contaminants in eggs are a much greater problem during the summer months.

Routine precautions and the following of the most careful techniques during harvesting should keep contamination at a minimum during these operations. Seed culture material should be constantly checked for bacterial sterility. Handling or prolonged incubation of the vaccine prior to final processing must be kept at a minimum to prevent the multiplication of the few contaminants that may be present. Additions of antibiotics to the products for reducing contamination is widely practiced. The presence of antibiotics or other bacteriostatic or bactericidal substances may interfere with tests designed to demonstrate bacterial contaminants in the finished product. This must always be considered in examining the final product. Further study shold be given to the use of Millipore filters to overcome the inhibitory effect of antibiotics in products that may be examined after the addition of these materials. It is presently required that culture tests for *Salmonella* be conducted by the manfacturer before the addition of antibiotics.

The presence of bacterial contaminants in eggs used for vaccine production would be self-limiting to some extent because many bacteria rapidly kill embryos or prevent their development. However, some embryos may not die as a result of contamination or may succumb in a death pattern very similar to that produced by the virus used for vaccine production. Under such circumstances it is quite possible for a few contaminated eggs to contaminate a whole batch of vaccine. More consideration has recently been given to the production of smaller lots of vaccine that can be tested separately for the degree and nature of any bacterial contamination before the final mixing.

Members of the veterinary biological products industry, control agencies, poultry pathologists, and the poultry industry have the same common interest and goal, namely, the production of poultry vaccines of utmost safety. If in the future, as in the past, it should be necessary to adopt some regula-

tion regarding bacterial contamination in virus vaccines of chick embryo origin, serious consideration should be given to the following points: 1. The establishment of a maximal permissable level of bacterial contamination; 2. The study of better ways of decreasing or preventing contamination of the products before or during production; 3. The development of improved testing procedures for detecting and identifying those contaminants in the vaccines that are likely to cause trouble in the field.

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THE DISTRIBUTION OF P^{32} AND S^{35} IN BRONCHITIS INFECTED EMBRYOS*

By P. P. LEVINE**
Department of Pathology and Bacteriology
New York State Veterinary College
Ithaca, New York

IN CONJUNCTION with Dr. R. Loos†, the electrophoretic migration patterns of neutralized and non-neutralized bronchitis virus harvested from chick embryos previously injected with radioactive phosphorus (P^{32}) were studied. It was noted that there was a marked difference in the radioactivity of the allanto-amnionic (AA) fluid from infected eggs and normal control eggs. This prompted further experimentation to investigate these results.

MATERIALS AND METHODS

Sodium radio phosphate solution (P^{32}) and radioactive sulfur (S^{35}) in the form of weak acid solution of SO_4 were procured from Abbot Laboratories and the Oak Ridge National Laboratories respectively. When necessary, the isotope solutions were sterilized in the autoclave. The Bickhart (N. Y.) strain of bronchitis virus was used to inoculate aseptically, the allantoic sac of chicken embryos in the usual way. The isotope solutions were similarly administered. The activity of the isotope solutions was adjusted so that no more than a total of 0.2 ml of fluid (isotope and virus or isotope and broth) were injected into each egg. The usual dose was 0.1 ml isotope solution and 0.1 ml virus.

† Fulbright Fellow, University of Ghent, Belgium .

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Fertile eggs of uniform size were employed in all of these studies. The radioactivity of the P^{32} was measured by bringing a known volume of the AA fluid to a total volume of 10 ml with water in a glass dish. This was centered under a thin mica end-window Geiger-Muller counter tube and counts were made in the usual manner. Embryos and other tissues were blotted, weighed, and "wet ashed"[1] in concentrated nitric acid. The radioactivity in the resultant clear solutions was measured as already described. The activity of the S^{35} in AA fluid was determined by drying 100 lambda in a thin, uniform layer in a ¾ inch diameter circle on a glass slide. This film was accurately centered under the Geiger counter and the counts made in the usual way. The radioactivity was recorded as counts per minute per ml of AA fluid or per gram of tissue. Only fluids and tissue from live embryos were used in procuring the data.

RESULTS

Some preliminary experimen were done with S^{35} and P^{32} and with incubation periods varying from 3 to 5 days. The age of the embryos varied from 9 to 11 days. The amount of radioactivity injected ranged from 0.02 millicuries to 0.4 millicurie. Results are given in table I.

The activity of the AA fluid from the control (non-infected) embryos was from 5.7 to 7.7 times the activity from the bronchitis infected embryos. When the intensity of radioactivity of the embryos was compared, the opposite situation occurred. The bronchitis embryos had absorbed from 6.4 to 32.2 times more radioactivity than the normals.

Since other preliminary experiments had indicated that the abnormal distribution of P^{32} in infected embryos occurred as early as 24 hours, experiment 5 was designed to gather data on shorter incubation periods. Eleven day embryos were inoculated with 0.1 ml bronchitis virus and 0.1 ml P^{32} containing 0.1 millicurie of activity into the allantoic sac. The radioactivity of the chorioallantoic membranes and the livers was determined in addition to the AA fluid and embryos. Pooled tissues and fluids from three or four embryos were examined for each incubation period. The histogram in figure 1 gives the results of this test.

TABLE I

The distribution of S^{35} and P^{32} in AA fluid and embryos infected with infectious bronchitis

Exp. No.	No. embryos	Age embryos in days	Inoculum	Incubation period in days	AA fluid av. ct/min/ml[1]	Embryo av. ct/min/gm[2]
1	4	11	0.4 mc S^{35}+Br	5	211,900	Not done
	4	11	0.4 mc S^{35}	5	1,219,000	
2	2	9	0.02 mc P^{32}+Br	4	1,500	48,330
	4	9	0.02 mc P^{32}	4	10,161	1,500
3	5	10	0.04 mc P^{32}+Br	3	4,640	39,820
	4	10	0.04 mc P^{32}	3	28,320	6,070
	5	10	0.08 mc P^{32}+Br	3	5,620	84,410
	3	10	0.08 m P^{32}	3	43,520	4,870
	4	10	0.1 mc P^{32}+Br	3	6,640	73,880
	4	10	0.1 mc P^{32}	3	40,470	11,530

1. av.ct/min/ml=average number of counts per minute per milliliter.
2. av.ct/min/gm=average number of counts per minute per gram.

Essentially no differences in P^{32} distribution in infected and normal embryos occurred through the eleventh hour of incubation. Beginning at 24 hours the activity of the virus bearing AA fluid was markedly less than that in the normal eggs. The situation was reversed in the embryo livers and the rest of the embryos where a marked concentration of P^{32} occurred in the infected tissues compared with the normal embryos. The infected CA membranes had significantly greater activity than the normals only at 24, 48 and 72 hours incubation.

It should be noted that no substantial weight differences between the infected and control embryos were noted until after 48 hours incubation. On the other hand, significant concentration of phosphorus took place in the infected embryos after 24 hours incubation.

DISCUSSION

The selective distribution of radioisotopes in infected

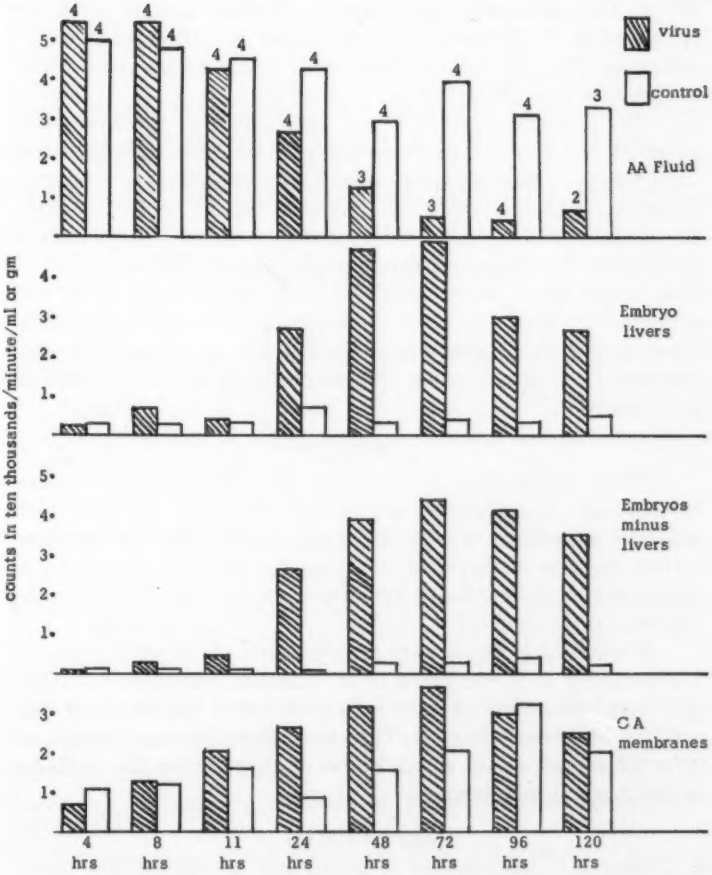

Fig. 1. The distribution of P^{32} in AA fluid, livers, embryos and chorio-allantoic membranes of bronchitis infected and normal 11 day embryos injected into the allantoic sac. Fluid and tissues were harvested after 4, 8, 11, 24, 48, 72, 96, and 120 hours incubation. The numbers over each block represents the number of embryos examined.

embryos is not confined to a single nuclide since S^{35} in the AA fluid of experiment 1 behaved in the same way as the P^{32} in the remaining experiments. Similar results with P^{32} in allantoic fluid have been described in influenza infected embryos although yellow fever virus and mumps virus failed to produce the effect[2].

The concentration of P^{32} in the tissues of the bronchitis infected embryos gives the virologist another objective tool with which to detect abnormalities in virus infected embryos. Other viruses cultivable in embryos should be investigated for similar phenomena. The method is amenable to quantitative determinations and the changes in phosphorus distribution might be a more sensitive and quicker test than the criteria now used for virus detection. Furthermore this technique might be of value in detecting the presence of agents growing in embryos when no other visible signs of infection are apparent.

SUMMARY

When radioactive sulphur S^{35} and phosphorus P^{32} were injected into the allantoic sac of chick embryos infected with infected bronchitis virus, the radioactivity became concentrated in the embryonic tissues. In non-infected control embryos the radioactivity remained for the most part in the allanto-amnionic fluid.

Normal AA fluid had a maximum of seven times the radioactivity of the infected fluid. Infected embryos and livers had maximums of 15 times and 13 times respectively the activity of normal tissues. The chorioallantoic membranes of infected embryos had a maximum of three times the activity of the normal membranes.

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ABSTRACTS OF PAPERS PRESENTED AT THE 28th ANNUAL MEETING OF THE LABORATORY WORKERS IN PULLORUM DISEASE CONTROL, JUNE 19 AND 20, 1956, NEWARK, DELAWARE

CASE REPORT——A PARATYPHOID OUTBREAK IN A POULTRY BREEDING FLOCK

C. I. Angstrom

N. Y. State Veterinary College, Regional Poultry Disease Laboratory, Kingston, N. Y.

Complete manuscript printed elsewhere in this issue.

STUDIES ON ERYSIPELAS IN TURKEYS

Clyde I. Boyer, Jr. and J. A. Brown

Department of Pathology and Bacteriology, N. Y. State Veterinary College, Ithaca, N. Y.

Complete manuscript printed elsewhere in this issue.

THE ISOLATION OF A VIRAL AGENT FROM CHICKENS SHOWING RESPIRATORY DISTRESS

M. S. Cover and W. J. Benton

University of Delaware Agricultural Experiment Station, Newark, Delaware

Complete manuscript printed elsewhere in this issue.

IMMUNITY STUDY OF THREE TYPES OF NEWCASTLE VACCINE

A. H. Dardiri, P. W. Chang and Dorothy E. Fry

University of Rhode Island, Kingston, R. I.

A controlled laboratory experiment was conducted to check the efficiency of three types of commercial Newcastle disease vaccines for

immunizing broilers during the growing period up to 12 to 16 weeks of age. The vaccines, purchased from a dealer, were water (Lasota strain), dust (B_1 strain), and killed Newcastle disease virus adsorbed by aluminum hydroxide (California strain 11914). Vaccination was administered according to the manufacturer's recommendaton to six groups of Rhode Island Red chickens totaling 340. The dams of these chicks had been vaccinated with Newcastle wing-web vaccine 7 months before the eggs were incubated.

The first and second group of 50 chicks each were vaccinated at five days with water and dust vaccine, respectively. The third and fourth group of 50 chicks each were vaccinated with these vaccines at 5 days and again at 4 weeks of age. The fifth group of 70 chicks was vaccinated at 10 days with the killed vaccine. The sixth group of 70 chicks was maintained as control. The serum of the 5-day-old chicks neutralized 100 units of G. B. Newcastle virus.

At 3 weeks of age and each week thereafter, five chickens were taken from each of the six groups, bled for hemagglutination inhibition (HI) test and challenged. The challenge was made by dropping 0.25 ml of 10^7 minimum embryo lethal dose pooled allantoic fluid of virulent virus into the trachea of each bird. Susceptibility was measured on the basis of appearance of either paralytic symptoms or death during a period of 8 weeks after challenge. The antibody response was measured by the beta type HI test. The groups that received a second dose of vaccine were treated similarly at six weeks of age.

The chickens that were vaccinated with one dose of water vaccine at 5 days of age had HI titers of 4 to 16 at 3 weeks. This titer dropped sharply at 4 weeks and only 10 percent of the birls had a titer of 4 to 8 when 4 to 12 weeks of age. Twenty eight percent of the birds survived challenge by the end of 12 weeks.

The chickens that were vaccinated with a dose of water vaccine at 5 days and again at 4 weeks of age had positive HI titers at 8 weeks and all the birds had positive titers of 4 to 64 up until the 15th week. Results on challenge indicated a 94 percent immunity.

Of the 50 chickens that were vaccinated with one dose of dust vaccine at 5 days, 34 chickens had HI antibody titers with a range of 4 to 64. Between the 6th and 9th week the titer was comparatively high and then started to drop. At 12 weeks only one bird out of five tested had a positive titer of 4. Results of challenge indicated that 70 percent of the birds were immune at 12 weeks.

Two doses of dust vaccine induced rising HI titers between the 7th and 14th week so that 42 percent of the chickens had a titer of 128. There was 100 percent resistance to challenge at 14 weeks.

The 70 birds in the group that was vaccinated with aluminum hydroxide adsorbed killed vaccine manifested a high HI antibody titer up until 12 weeks of age. The survival to challenge to the end of 16 weeks of age was 98.6 percent. In the control group none of the chicks had a positive HI titer at the end of the experiment.

The survival of ND virus in the chickens' environment after vaccination from dust and water vaccines was tested. It was possible to recover ND virus in the chickens' environment from the water and dust vaccine at 36 and 24 hours respectively.

The results of this experiment showed that administration of one dose of water or dust vaccine did not protect the broiler through 12 weeks of age. Two vaccinations with water or dust vaccine administered at 5 days and again at 4 weeks produced adequate immunity at 16 weeks. These results were comparable to the administration of one dose of killed vaccine at 10 days.

A MODIFIED CHICKEN EMBRYO INOCULATION TECHNIQUE FOR THE ISOLATION OF VIRUSES FROM THE RESPIRATORY TRACT OF CHICKENS

Julius Fabricant
Department of Pathology and Bacteriology, N.Y.S. Veterinary College, Ithaca, N. Y.

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TISSUE CULTURE STUDIES OF AVIAN VIRUSES

J. E. Fahey
Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

The purpose of this study was to investigate the growth of some of the more commonly occurring avian viruses, such as infectious bronchitis virus (IBV), in tissue culture and to determine what distinguishing characteristics, if any, they possessed under such circumstances. All techniques employed were standard tissue culture procedures. The strains of IBV used were the Connaught Laboratories vaccine strain R and the Beaudette embryo-passed strain. The results of these studies have been published in full elsewhere (Fahey, J. E. and J. F. Crawley., Propagation of infectious bronchitis virus in tissue culture. Canad. Jour. Microbiol., *2*, 503, 1956).

Initially, serial passage of the IBV strains in minced chorioallantoic membrane in stationary flask cultures indicated that this virus did propagate. The Beaudette strain was, for example, passaged serially under these conditions 10 times. The final inoculum represented a dilution of greater than 10^{-26} of the original inoculum.

Growth of both the R and Beaudette strains in stationary flask cultures were studied. In both instances the absorption period was from 4 to 8 hours and resulted ultimately in the complete disappearance of egg-infective virus. The latent period was 8 to 12 hours for the Beaudette strain and 12 to 16 hours for the R strain.

It was demonstrated that IBV did not produce a cytopathogenic effect (CPE) when grown in stationary tube cultures of chick embryo heart fibro-blasts. The virus was produced by such cell cultures as long as cellular growth could be maintained, which in some cases was for 20 days. There was no alteration in the rate of change of pH in the infected cultures compared to controls. This is quite different from the results obtained with Newcastle virus where complete destruction of chick embryo cells occurs in 3 to 5 days.

Attempts were also made to propagate IBV in other cell lines, and it was found that monkey kidney cells were capable of supporting the propagation of these strains. Virus was produced by a monolayer of monkey kidney cells over a period of 30 days, the LD^{50} of culture fluids ranging from $10^{-2.9}$ to $10^{-4.8}$. There was no CPE observed in monkey kidney cells infected with and producing IBV. The similarities between growth of IBV in chick embryo and monkey kidney cells and the lysogenic system in bacteria was noted and discussed.

PRELIMINARY INVESTIGATIONS ON THE HEMATOLOGY OF BROILER FLOCKS

T. N. Fredrickson, H. L. Chute and D. C. O'Meara

Department of Animal Pathology, Maine Agricultural Experiment Station, University of Maine, Orono, Maine

Complete manuscript printed elsewhere in this issue.

A SIMPLIFIED METHOD FOR THE TITRATION OF ANTIGEN IN THE BETA HEMAGGLUTINATION INHIBITION TEST

R. F. Gentry

Department of Veterinary Science, Pennsylvania State University, University Park, Pennsylvania

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DOSAGE RESPONSE OF TRACHEAL MUCOSA TO INFECTIOUS BRONCHITIS VIRUS

Erwin Jungherr, T. W. Chomiak, and R. E. Luginbuhl

Department of Animal Diseases, Storrs (Connecticut) Agricultural Experiment Station, Storrs, Connecticut

A study was made of the pathogenesis and dosage response of uncomplicated infectious bronchitis in the trachea of chickens inoculated with known minimum embryo infective doses (MEID). Small groups of 7 to 11 week-old chickens, reared in isolation and known to be free from infectious bronchitis (IB), Newcastle disease and PPLO, were inoculated intratracheally with 0.2 ml of tryptose broth dilutions of field vaccine strain #82828, 8th embryo passage. The birds were maintained in modified Horsfall-Bauer units, and one bird each was killed every third day over a period of 21 days for determination of IB virus neutralizing doses and histopathologic changes in the trachea. In infectious bronchitis the tracheal mucosa undergoes cyclic changes for 18 to 21 days: an acute phase, 1 to 3 days, characterized by epithelial hypertrophy and marked edema; a reparative phase 6 to 9 days, characterized by epithelial hyperplasia and marked cellularity of the lamina propria; and an immune phase, 12 to 18 days, characterized by restoration of the epithelium and either follicular or mild follicular or mild focal diffuse lymphoid infiltration of the propria. Under the conditions of these exepriments, where the strain remained constant, the onset of symptoms, histologic changes, and demonstrable neutralizing antibodies varied primarily with dosage. Low dosages (0.1 to 1 MEID) delayed the reactions and decreased their intensity so that the acute histologic phase was almost skipped. High dosages (10^2 to 10^6 MEID) accelerated the reactions and increased their intensity. Dosage levels of 10^4 produced extreme reactions, which were not intensified by levels of 10^6, indicating that the limits of reactive capacity had been reached. Of particular interest was the observation that disease was induced occasionally with doses as low as one-tenth MEID, which fact suggested a higher susceptibility of chickens to IB virus, as compared to embryos obtained from immune stock.

DISTRIBUTION OF P^{32} AND S^{35} IN BRONCHITIS INFECTED CHICK EMBRYOS

P. P. Levine

Department of Pathology and Bacteriology, N.Y.S. Veterinary College, Ithaca, N. Y.

Complete manuscript printed elsewhere in this issue.

EGG TRANSMISSION OF THE CHRONIC RESPIRATORY DISEASE AGENT—PRELIMINARY REPORT

Olga M. Olesiuk and H. Van Roekel

Department of Veterinary Science, University of Massachusetts, Amherst, Mass.

Egg transmission of the chronic respiratory disease (CRD) agent was investigated under controlled laboratory conditions. Among a group of 480 White Leghorn breeders, 76 dams were selected on the basis of producing embryos and day-old chicks manifesting CRD lesions. These 76 dams were segregated and mated to form a breeding unit for further egg transmission studies. All eggs were incubated and chicks were brooded at the laboratory under controlled conditions. A total of 7,353 eggs was incubated in 18 different settings. The embryos examined revealed that among 519 unpipped eggs 4.2 percent were positive for CRD lesions and among the 887 pipped eggs 17.1 percent were positive. A total of 3,986 chicks was examined immediately after hatching of which 7.5 percent were positive.

Chicks from six different hatches were reared in isolation to 8 weeks of age. At 4 weeks of age the chicks from each hatched group were divided into two equal groups; one group was stressed with infectious bronchitis virus, and the other group was left unstressed. In the six hatches a total of 364 chicks was stressed and symptoms of CRD were observed in chicks from two hatches. Gross lesions of CRD were noted in chicks from five of the six hatches. Follow-up studies by chicken and turkey inoculations yielded positive evidence of CRD in three hatches and questionable evidence in a fourth hatch. In the unstressed groups (325 chicks), none of the chicks revealed conclusive evidence of CRD.

In a later study, which is still in progress, two different groups of White Leghorn dams were selected on the basis of giving a positive agglutination reaction with the rapid serum plate test with an antigen prepared from the pleuropneumonia-like organism causing CRD. The two groups of dams were maintained in separate buildings and one group was treated with antibiotics (streptomycin injections and aureomycin in the feed). The procedure of study in the hatches produced was similar to that of the previous study except that different stressing mechanisms were applied. Data obtained from the first eight hatches revealed a lower incidence of CRD lesions in the embryos and cull chicks than observed in earlier experiments. Completed studies on three of the four hatches reared revealed suspicious CRD lesions in two hatches but these were negative by bird inoculation. In comparing the results of the treated and untreated groups, no apparent benefit from the antibiotic treatment could be detected in the embryos and chicks from the treated group.

PRELIMINARY BLOOD STUDIES IN CHICKENS WITH A SYNOVITIS CAUSED BY THE INFECTIOUS SYNOVITIS AGENT, PLEUROPNEUMONIA-LIKE ORGANISMS AND A COMBINATION OF THE TWO AGENTS

N. O. Olson, D. C. Shelton, D. A. Munro and Ruth Bletner

West Virignia University, Morgantown, West Virginia

Complete manuscript printed elsewhere in this issue.

A DOUBLE SERUM NEUTRALIZATION TEST TO INDICATE THE RELATIVE LIVE VIRUS CONTENT OF INFECTIOUS BRONCHITIS VACCINE

D. C. O'Meara

Department of Animal Pathology, University of Maine, Orono, Maine

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PROPAGATION OF NEWCASTLE DISEASE VIRUS IN TISSUE CULTURE PREPARATIONS

E. H. Seadale and R. W. Winterfield

Department of Veterinary Science, University of Massachusetts, Amherst, Mass.

Two strains of Newcastle disease virus (NDV)—M9251 and Roakin— were propagated through 100 generations in Maitland and trypsinized chick-embryo tissue culture preparations. Satisfactory NDV reproduction was obtained with both systems, although highest titers were often found in Maitland cultures. When assayed in embryonated chicken eggs, titers up to $10^{8.5}$ per ml were recorded. Growth curves of the two strains were similar with maximum virus yields obtained between 40 and 48 hours. The log phase of reproduction was generally entered between 6 and 12 hours. Hemagglutinating activity of tissue culture propagated NDV was evident in low level titers.

Maximum titers were obtained when the fluid volume in 32 ounce prescription bottles was from 20 to 30 ml or when the fluid depth was not more than 1.25 cm. Titers were progressively lower with greater quantities. A high concentration of chick embryo extract increased trypsinized tissue growth considerably in a relatively short time, but a significant increase in virus growth was difficult to demonstrate when titrated by normal procedures in chicken embryos.

More satisfactory cell cultures were established and maintained when calf serum was employed rather than horse serum in starting and replacement mediums. Horse serum occasionally proved toxic for the cells. Cultures were maintained for an extended period at 31 C with a reduced serum level. Cell destruction was less in roller tubes than in stationary cultures where the preparation was maintained for more than a week.

Titrations of NDV pools based on cytopathogenic effects in roller tube tissue cultures were slightly more sensitive than assays in chick embryos. No change in virulence of the two strains occurred for either chickens or chick embryos.

STUDIES OF INFECTIOUS SYNOVITIS

M. Sevoian, G. H. Snoeyenbos, H. Basch, and I. Reynolds

Department of Veterinary Science, University of Massachusetts, Amherst, Mass.

A series of controlled experiments were conducted to investigate the pathology and control of infectious synovitis. Chicks and poults, 4 weeks of age, were injected intravenously with an infective agent isolated originally from a field outbreak in turkeys.

The thrombocyte, heterophil, and total leukocyte counts and sedimentation rate increased. The hematocrit, hemoglobin content, red blood cell count, and lymphocyte count (differential) decreased. The blood disturbances were most severe between the 5th and 16th days post inoculation.

Vascular endothelial thickening and adventitial proliferation were seen in the cerebrum, cerebellum, and optic lobe. There was degeneration of some of the Purkinje cells. Occasionally, cerebellar lesions similar to those of encephalomalacia were seen. Gross enlargement of the liver was seen as early as the 5th day post inoculation. Perivascular, periportal and interparenchymal cellular hyperplasia within 5 days post inoculation was seen microscopically. Mitosis was seen in some of these cells. Because of their morphology, location, and mitotic activity, these cells were interpreted to be reticular cells of the reticulo-endothelial system. There was cellular hyperplasia along the connective tissue framework of the heart. These cells were morphologically similar to those described in the liver. Less frequently, focal mononuclear infiltration and necrosis of the myocardium and a fibrinous inflammation of the pericardium were seen. Cellular hyperplasia was associated with the connective tissue of the gizzard. These cells were morphologically similar to those described in the liver. There was a gross enlargement of the spleen beginning about the 5th day post inoculation. Sinusoidal areas appeared decreased as a result of a reticular cell or lymphocytic hyperplasia, or both. Approximately 5 days after inoculation, there was a granulocytic hyperplasia in the bone marrow which was gray on gross appearance. The synovial cavities of joints contained inflammatory exudate. Synovial membranes were thickened. Articular cartilage had little or no evidence of inflammation. Atrophy of the thymus and the bursa of Fabricius resulted from a lymphoid degeneration in the medulla and cortex. Interlobular septa of the lungs were thickened as a result of cellular proliferation. These cells were morphologically similar to those described in the liver. There was a predominantly heterophilic inflammation involving the sternal bursa and adjacent subcutaneous tissue.

Prophylactic medication at 500 gm-per-ton levels in feed of bacitracin, streptomycin sulfate, procaine penicillin, and para-aminobenzoic acid failed to exert appreciable protection for either chickens or turkeys. Triple sulfa medication (Whitmoyer) administered continuously in the water at the rate of 2 oz per gallon also resulted in no appreciable protection. Subcutaneous injection of oxytetracycline in oil (100 mg) at time of infection gave partial, transient protection in both species. Essentially complete protection was afforded by chlortetracycline at a 200 gm-per-ton level in the feed during prophylactic medication of both chickens and turkeys. Partial protection was demonstrated by levels as low as 50 gm per ton. Parallel trials with Furazolidone showed less consistent results with chickens. Some activity was demonstrated at

levels of 100 and 200 gms per ton, and complete protection was usually obtained at 400 gms per ton. Only slight prophylactic activity was demonstrated by Furazolidone at levels of 400 gms per ton fed to turkey poults. Treatment at onset of symptoms (5 to 7 days post inoculation) at levels of 200 and 400 gms of chlortetracycline per ton for 14 days decreased the severity and extent of the lesions in chickens and turkeys. Less improvement as compared to controls was noted by similar use of Furazolidone in chickens. Levels as high as 800 gms of either chlortetracycline or Furazolidone per ton fed for 14 days after infection were effective in preventing symptoms or lesions in chickens during the period of medication, but did not prevent appearance of the disease after drug withdrawal.

OBSERVATIONS ON STRESS FACTORS AND SEROLOGICAL RESPONSE IN SALMONELLA TYPHI-MURIUM INFECTION IN CHICKS

J. McN. Sieburth and E. P. Johnson
Animal Pathology Section, Virginia Agricultural Experiment Station, Blacksburg, Va.

Paratyphoid infections, especially those associated with *Salmonella typhi-murium*, can be a cause of early chick losses and may become a serious flock problem to the hatcheryman. This report deals with some preliminary results on the pathogenicity of *S. typhi-murium* for the day-old chick, the effect of concurrent infections, the effect of nitrofurazolidone on the serological response of infected chicks and the development of an indirect hemagglutination test for the detection of *Salmonella* in poultry.

Orally administered *Salmonella typhi-murium* appears to be very pathogenic for the day-old chick, with 100 percent infection arising from $10^{2.0}$ and 50 percent mortality from $10^{3.5}$ viable organisms per chick. Concurrent infections appear capable of either aggravating (bluecomb agent) or alleviating (Crawley's agent) *S. typhi-murium* infection of chicks. Although nitrofurazolidone did not appreciably reduce the incidence of liver or intestinal infection, serological response was apparently prevented. The widespread use of NF-180 may be of importance in evaluating serological testing. The indirect hemagglutination test which has been developed, not only detects a serological response earlier than the agglutination test but gives a titre two to five times greater. Chicken erythrocytes, modified by four *Salmonella* serotypes (group specific but not species specific), gave polyfalent, indirect, hemagglutination titres in good agrement with the species specific indirect hemagglutination test. The polyvalent indirect hemagglutination test may be of value as a screening test for the detection of *Salmonella* infections of poultry.

SOME FACTORS INFLUENCING THE IMMUNE RESPONSE FROM ADMINISTRATION OF NEWCASTLE DISEASE VACCINE THROUGH DRINKING WATER UNDER LABORATORY AND FIELD CONDITIONS

R. W. Winterfield and E. H. Seadale

Department of Veterinary Science, University of Massachusetts, Amherst, Mass.

Seven laboratory trials with more than 700 chickens and 18 field

flocks, totaling 59,100 birds including suitable controls, were employed in the investigation. Three strains of Newcastle disease virus (NDV)—B1, LaSota, and F—were used as vaccines.

Laboratory trials with B1 NDV demonstrated the necessity of having vaccine titers of at least $10^{4.5}$ to $10^{5.0}$ e.i.d. $_{50}$ per ml of drinking water to obtain a satisfactory immune response. In field trials where divided or split flocks were available for vaccination, the greatest immune response consistently occurred when the vaccine titer in the drinking water was the highest. Where titers of 10^4 and below were present, the HI and SN antibody response was essentially negative, while 10^6 to 10^7 provided the best antigenic stimulus.

Eight flocks comprising 22,700 birds, were vaccinated at 4 days to 2 weeks of age with B1 NDV. Six flocks responded unsatisfactorily. In the latter flocks, 30 percent or less of the serums tested 4 to 7 weeks post-vaccination were SN positive (neutralization index of 2.0 or more). The two remaining flocks yielded sera of which 60 and 75 percent were SN positive. Parentally conferred ND antibody levels were high at the time of vaccination in those flocks responding poorly. Vaccination of 3 flocks that were 3 weeks old and devoid of congenital antibody resulted in satisfactory immunity when tested 6 weeks later.

LaSota and F strains induced satisfactory immunity in field flocks when the latter were vaccinated after passive immunity had subsided. Vaccination in the presence of passive immunity resulted in an erratic, and often poor, immune response.

Chickens vaccinated under laboratory and field conditions with LaSota NDV showed higher serologic evidence of immunity but more clinical respiratory signs after primary vaccination than those vaccinated with F and B1 NDV. The latter two strains provoked little or no apparent respiratory reaction. However, all three strains were relatively avirulent; none appeared neuropathogenic. The spreading potential was somewhat greater with the LaSota strain than with the other two.

The gastrointestinal tract of chickens proved relatively refractory to infection by B1, LaSota, and F NDV strains when instilled in the crop. A double capsule containing 10^6 e.i.d.$_{50}$ of vaccine was used to prevent contamination of the upper respiratory tract. However, birds vaccinated through the drinking water with a comparable dosage from the same vaccine virus pool showed evidence of infection. In the latter case, infection by these NDV strains was presumably initiated via the respiratory mucosa rather than the gastrointestinal tract at the dosage level given.

LABORATORY ANALYSIS OF SEVERAL FIELD OUTBREAKS OF CHRONIC RESPIRATORY DISEASE

J. F. Sulivan, E. Gill, A. Somer, and K. Heddleston
Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

Complete manuscript printed elsewhere in this issue.

A COMPARISON OF FOUR IN VITRO METHODS FOR THE ISOLATION OF THE PLEUROPNEUMONIA-LIKE ORGANISM OF CHRONIC RESPIRATORY DISEASE FROM TRACHEAL EXUDATE

J. R. E. Taylor, Julius Fabricant, and P. P. Levine

Department of Pathology and Bacteriology, New York State Veterinary College, Ithaca, N. Y.

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BACTERIAL CONTAMINANTS IN POULTRY LIVE VIRUS VACCINES OF EGG EMBRYO ORIGIN

J. E. Williams

Program Services Section, Animal Disease Eradication Branch, Agricultural Research Service, Washington, D. C.

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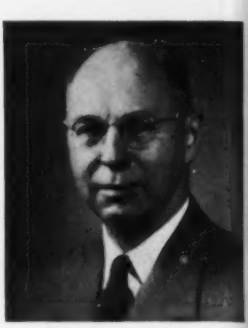

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In Memoriam

Dr. Frederick R. Beaudette

1898 - 1957

Chairman, Department of Animal Pathology
Rutgers University
New Brunswick, N. J.

From the research laboratories of the late Dr. Frederick R. Beaudette, have stemmed the most notable achievements in the history of preventive poultry medicine.

Throughout his entire career, this scientist served as the mentor and ennobling inspiration for our own efforts and contributions to the poultry industry of America and the world.

The untimely passing of Dr. Beaudette has created an immeasurable void for those engaged in poultry production. But, from his findings, and the pattern that he established in fervid and unilateral consecration to animal pathology, we at Vineland Poultry Laboratories take heart.

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Dr. Arthur Goldhaft, V.M.D.
Director, Vineland Poultry Laboratories

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